PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K

A2

(11) International Publication Number: WO 98/16185

(43) International Publication Date: 23 April 1998 (23.04.98)

(21) International Application Number: PCT/US97/18710

(22) International Filing Date: 17 October 1997 (17.10.97)

(30) Priority Data:
08/733,131 17 October 1996 (17.10.96) US

(71) Applicant: NPS PHARMACEUTICALS, INC. [US/US]; Suite 240, 420 Chipeta Way, Salt Lake City, UT 84108 (US).

(72) Inventors: SANGUINETTI, Michael, C.; 4922 N. East Meadows Drive, Park City, UT 84060 (US). MUELLER, Alan, L.; 2510 E. Skyline Drive, Salt Lake City, UT 84108 (US).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

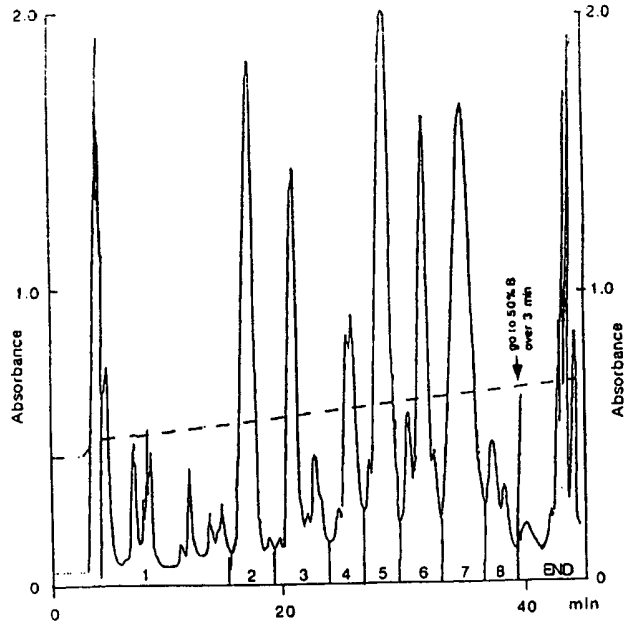
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: POTASSIUM CHANNEL BLOCKING COMPOUNDS AND THEIR USE

(57) Abstract

Specific potent transient outward potassium channel inhibitors, and polypeptides derived from spider venom are described. Also described are methods of screening for agents specifically active on transient outward potassium channels or other types of potassium channels, methods of treating a disease or condition by administering an agent active on a transient outward potassium channel or certain agents active on other potassium channels, and methods of using a potassium channel inhibitor derived from spider venom as an insecticidal agent.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

DESCRIPTION

POTASSIUM CHANNEL BLOCKING COMPOUNDS AND THEIR USE

RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application Sanguinetti et al., U.S. Serial No. 08/213,742, filed March 14, 1994, entitled SCREENING METHODS FOR IDENTIFICATION OF SPECIFIC POTASSIUM CHANNEL INHIBITORS, which is a continuation-in-part of Sanguinetti et al., U.S. Serial No. 08/033,388, filed March 18, 1993, entitled POTASSIUM CHANNEL BLOCKING COMPOUNDS AND THEIR USE, now abandoned, which are incorporated by reference herein, including drawings.

Field of the Invention

This invention relates to inhibitors of the transient outward potassium channel and other potassium channels.

Background of the Invention

The following is a description of relevant art, none of which is admitted to be prior art to the claims.

Potassium (K^+) channels are membrane-spanning proteins that allow the selective movement of K^+ into or out of cells in response to changes in membrane potential, or in response to activation by cations and/or ligands. The primary role of K^+ channels is maintenance of the resting membrane potential; another role concerns their contribution to repolarization of action potentials in excitable cells. Potassium channels represent a diverse group of ion channel proteins, and several toxins have been described that act primarily (if not exclusively) by blocking one or more specific K^+ channels (Rudy, "Diversity

and Ubiquity of K Channels", *Neuroscience* 25:729, 1988). Cardiac cells are characterized by a remarkable variety of different K^+ channel subtypes. Several K^+ channel types are opened in response to depolarization of the membrane during an action potential, and the currents carried by these different channels sum to cause repolarization of the membrane to the resting potential. One of these channel types, the transient outward K^+ channel, conducts a current (I_{to}) which activates very rapidly (within 1-10 milliseconds) upon membrane depolarization and then decays (inactivates) rapidly (10-200 milliseconds). I_{to} contributes most significantly to initial repolarization of the cardiac action potential, and is blocked by several non-specific pharmacological compounds such as aminopyridines and tedisamil, a class III antiarrhythmic agent (Dukes et al., "Tedisamil Blocks the Transient and Delayed Rectifier K^+ Currents in Mammalian Cardiac and Glial Cells", *J. Pharmacol. Exp. Ther.* 254:560, 1990). Blockage of I_{to} leads to prolongation of the cardiac action potential. I_{to} has been recorded from human cardiac cells as described, for example, in Escande et al., "Two Types of Transient Outward Currents in Adult Human Atrial Cells", *Am. J. Physiol.* 252:H142, 1987. Lengthening of cardiac action potentials (and hence refractoriness) is said to be one mechanism for suppression of reentrant atrial and ventricular arrhythmias (Lynch et al., "Therapeutic Potential of Modulating Potassium Currents in the Diseased Myocardium", *FASEB J.* 6:2952, 1992). Currently available class III antiarrhythmic agents, most of which act by blocking a separate subtype of K^+ channels known as delayed rectifier K^+ channels, may cause excessive prolongation of cardiac action potentials that can lead to the development of a ventricular arrhythmia, known as torsades de pointes (Sanguinetti, "Modulation of Potassium Channels by Antiarrhythmic and Antihypertensive Drugs", *Hypertension* 19:228,

1992).

The opening of voltage-dependent K^+ channels is also the mechanism by which repolarization of the cell membrane occurs during the very short action potential characteristic of central neurons. Transient outward K^+ currents (referred to as I_A in neurons) play a role in this process. Dendrotoxin, a toxin derived from snakes, selectively blocks the delayed non-inactivating K^+ current in dorsal root ganglion neurons (Penner et al., "Dendrotoxin: A Selective Blocker of a Non-inactivating Potassium Current in Guinea-pig Dorsal Root Ganglion Neurones", *Pflugers Arch.* 407:365, 1986), and also blocks the transient outward K^+ current (I_A) in hippocampal slices (Halliwell et al., "Central Action of Dendrotoxin: Selective Reduction of a Transient K Conductance in Hippocampus and Binding to Localized Acceptors", *Proc. Natl. Acad. Sci. USA* 83:493, 1986). Prolongation of action potential duration in neurons by dendrotoxin results in an enhanced release of neurotransmitters (Harvey and Anderson, "Dendrotoxins: Snake Toxins That Block Potassium Channels and Facilitate Neurotransmitter Release", *Pharmac. Ther.* 31:33, 1985). It has been suggested that further work in this field may confirm this as a pharmacological approach to the treatment of cognitive disorders such as Alzheimer's disease (Lavretsky and Jarvik, "A Group of Potassium-channel Blockers-Acetylcholine Releasers: New Potentials for Alzheimer Disease? A Review", *J. Clinical Psychopharm.* 12:110, 1992).

Summary of the Invention

This invention generally concerns specific and potent novel inhibitors or blocking agents of the transient outward potassium channel, for example, currents referred to as I_{to} or I_A in cardiac cells and in neurons,

respectively, or of a subset of types of transient outward potassium channels. The invention also features novel polypeptides isolated from spider venom, or their equivalent, which are active at one or more potassium channels, *e.g.*, the transient outward potassium channel.

Specifically, examples are provided of the novel activity of polypeptide toxins isolated from the venom of the spiders *Heteropoda venatoria*, and *Olios fasciculatus* These peptides, referred to herein simply as Compounds 1, 2, 3, and 4 are examples of specific and potent blockers of voltage-dependent transient outward K^+ channels, which block the corresponding whole-cell current (I_{to}) of cardiac cells. As such, these toxins, fragments thereof, or compounds discovered using a ligand binding assay (or its equivalent) employing these toxins, fragments, or their equivalent, are useful in the treatment of cardiac arrhythmias and have utility in the treatment of disorders of learning and memory, (such as Alzheimer's disease), Parkinson's disease, multiple sclerosis, schizophrenia, epilepsy, stroke and muscle spasticity. Further, such agents are useful as reagents in analyzing the distribution and function of particular types and subtypes of potassium channels.

In general, useful K^+ channel inhibiting polypeptides of the type described and claimed herein can be isolated from the venom of spiders, for example, of the spiders *Heteropoda venatoria* and *Olios fasciculatus*. Other polypeptides (or their equivalent) with similar or homologous amino acid (or other compound or monomer) sequences that block potassium channels can also be isolated. The determination of the existence of such K^+ channel blocking activity in toxins derived from spider venom thus demonstrates that it is useful and productive to screen for other such polypeptides in spider venom. This invention also concerns methods of using these

polypeptides to screen for other agents acting at a common site (*i.e.*, the transient outward potassium channel) as active agents.

Chemical agents that selectively block I_A channels in central neurons, and thereby cause enhanced release of neurotransmitters, are believed to be useful in the treatment of Alzheimer's disease and other neural disorders. Similarly, agents which selectively block I_{to} channels in cardiac cells are believed to be useful in treatment of cardiac arrhythmias. Thus, these polypeptides and related compounds or agents can be used for the treatment of cardiac arrhythmias, Alzheimer's disease, Parkinson's disease, multiple sclerosis, schizophrenia, epilepsy, stroke and muscle spasticity.

Thus, in a first aspect, the invention features specific potent transient outward potassium channel inhibitors, blockers or antagonists.

The phrase "transient outward potassium channel" is a well recognized phrase which defines a specific subtype of potassium channels within a variety of cells, *e.g.*, as characterized by the currents I_{to} and I_A noted above, in cardiac and neural cells, respectively. Subtypes of transient outward potassium channels have been cloned, isolated, and identified (*e.g.*, Kv1.4, Kv4.2, and Kv4.3).

The term "inhibitor" is used to mean agents which reduce current conducted by transient outward potassium channels. In the art, terms such as "blocker" and "antagonist" have been used interchangeably with the term "inhibitor".

The term "specific" means that the blockage of transient outward K^+ channels is half-maximal (IC_{50}) at or below 100 nM, and that no effect on currents of other K^+ channels (such as delayed rectifier, inward rectifier, acetylcholine-activated or ATP-inhibited K^+ channels), Na^+

or Ca^{2+} channels occurs at concentrations at least 10-fold greater than the IC_{50} for transient outward K^+ channels.

By "potent" is meant that a given transient outward K^+ channel is blocked by 50% at a concentration of an inhibitor less than 100 nM, more preferably less than 10 nM, and even more preferably less than 1 nM.

In preferred embodiments, these agents are polypeptides or are derived from polypeptides present in spider venom. While specific examples of such polypeptides are provided herein, these examples are not limiting in this invention and those of ordinary skill in the art will recognize that other polypeptides can be readily identified within various spider venoms, including but not limited to those described herein. In addition, those of ordinary skill in the art will recognize that equivalent polypeptides can be synthetically formed using standard procedures. Specific portions of those peptides which are active in blocking, inhibiting, or antagonizing a selected transient outward potassium channel can be readily identified using standard screening procedures. For example, specific peptide fragments can be synthesized, or produced from intact polypeptides using various peptidases, and those fragments assayed for inhibitory activity in assays, as described below. Those fragments which are active as inhibitors are useful in this invention in various screening assays, and in therapeutic applications.

In addition, analogues or muteins of such polypeptides can be readily synthesized. These may contain modifications in the amino acid sequence in regions which do not affect the inhibitory or blocking activity of the original polypeptide. In more conserved regions of the inhibitor, amino acids may be substituted such that the activity of the inhibitor is not significantly altered, for example, by substitution of small amino acids, such as glycine, for other small amino acids, such as valine, or

positively or negatively charged amino acids for similarly charged amino acids.

The term "derived" indicates that compounds can be synthesized based upon the general structure of polypeptides identified in spider venom. Such derivatization is performed by methods well recognized in the art, specific examples of which are generally provided above. Preferably, the term "derived" includes analogues, muteins and fragments, as described above, which have a desired modulatory activity of a polypeptide toxin described herein.

The above inventions are exemplified by polypeptides found in the venom of the spiders *Heteropoda venatoria* and *Olios fasciculatus*. Four specific polypeptides of this invention and the fractions in which they are present according to this invention include *Heteropoda venatoria* peptide Compound 1 (SEQ. ID. NO. 1), *Heteropoda venatoria* peptide Compound 2 (SEQ. ID. NO. 2), *Olios fasciculatus* peptide Compound 3 (SEQ. ID NO. 3), and *Heteropoda venatoria* peptide Compound 4 (SEQ. ID. NO. 4). In one example, polypeptides of this invention block transient outward K^+ channels in cardiac and neural cells. This invention includes polypeptides which have substantially the same amino acid sequence, and substantially the same K^+ current blocking activity as the polypeptides, *Heteropoda venatoria* peptides Compound 1 (SEQ ID NO. 1) and Compound 2 (SEQ ID NO. 2), *Olios fasciculatus* peptide Compound 3 (SEQ ID NO. 3), and *Heteropoda venatoria* peptide Compound 4 (SEQ ID NO. 4), and.

In a second aspect, the invention features a method for screening for a transient outward potassium channel active agent by contacting a transient outward potassium channel with a known specific transient outward potassium channel inhibitor (such as those described

above) and a potential transient outward potassium channel active agent, and detecting inhibition of binding of the known inhibitor by the potential active agent. Inhibition of binding is an indication of a useful transient outward potassium channel active agent. Such active agents can be readily screened to determine their specificity. In a preferred embodiment, the known specific transient outward potassium channel inhibitory agent is active on a subset of types of transient outward potassium channels. For example, an agent may be active on a Kv4.2 channel, but not on a Kv1.4 channel, or an agent may be active on a Kv1.4 channel, but not on a Kv4.2 channel. Such selectivity also applies to other types of transient outward potassium channels which may be identified.

An "active agent" is a compound that either increases (if it acts as an agonist) or decreases (if it acts as an inhibitor) current through a transient outward potassium channel.

Reference to a "subset" of types of transient outward potassium channels in this context indicates that an agent is active on one or more of the types of transient outward potassium channels (e.g., Kv1.4, Kv4.2, and Kv4.3), but not on all such channels. Thus, the agent is selectively active on these different channels.

In a related aspect, this invention provides a method for screening for agents active on the Kv4.2 or Kv4.3 potassium channels. This method involves detecting competition for binding of a labeled compound known to be active on these channels by a potential agent active on such channels. In a preferred embodiment, the Kv4.2 or Kv4.3 channels are expressed in oocytes, *e.g.*, *Xenopus* oocytes. Also in a preferred embodiment, the compound known to be active on the Kv4.2 or Kv4.3 channels is not active on the Kv1.4 channels. Examples of such compounds include Compounds 1 and 2.

In another related aspect, the invention features a method for screening spider venom for a useful K^+ channel active agent as exemplified by methods described herein. Such venom is screened to determine fractions which contain the desired activity.

By "K^+ channel active agent" is meant a compound that either increases or inhibits any other type of K^+ channel such as delayed rectifier, inward rectifier, Ca^{2+}-activated or ATP-sensitive K^+ channels.

In preferred embodiments, the screening method involves use of transient outward potassium channels from cardiac or neural tissue.

Also within the scope of this invention is a method for identifying compounds that bind to the transient outward K^+ channel, preferably at the same site as that bound by one of the *Heteropoda venatoria* peptides Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), or Compound 4 (SEQ ID NO. 4), or *Olios fasciculatus* peptide Compound 3 (SEQ ID NO. 3). Compounds 1, 2, 3, and 4 may bind to the same site, different sites, or overlapping sites on a transient outward K^+ channel, however it is likely that the differences in binding sites are small. In this context, "binding" refers to an interaction between a compound (such as one of Compounds 1, 2, 3, or 4) and a site on a K^+ channel (such as a transient outward potassium channel). A binding site generally is formed by a number of amino acid residues of the channel, which together form a set of contact points and/or a space or pocket for locating and/or interacting with the bound compound.

In another aspect, the invention features a method for treatment of a disease or condition in which a therapeutically useful result is achieved by modulating a transient outward potassium channel activity, by administering to the organism a therapeutically effective amount

of a specific transient outward potassium channel inhibitor, or a polypeptide (or its equivalent) from spider venom. Specific diseases to be treated include (but are not limited to) those listed above.

In a preferred embodiment, the treatment involves modulating the activity of Kv4.2 or Kv4.3 or Kv1.4 potassium channels by administering a therapeutically effective amount of a compound active on such a channel.

By "modulating" is meant a decrease of transient K^+ channel activity, for example, by blocking the pore of the channel, or by changing the voltage dependance of channel gating.

Treatment involves the steps of first identifying a patient (human or non-human) that suffers from a disease or condition by standard clinical methodology and then providing such a patient with a therapeutically effective composition of the present invention.

By "therapeutically effective" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of the disease or condition. Generally, it is an amount between about 1 nmole/kg and 1 μmole/kg of the molecule, dependent on its EC_{50} and on the age, size, and disease associated with the patient.

In a further aspect, the invention features a pharmaceutically acceptable composition including a specific transient outward potassium channel inhibitor or spider venom polypeptide (or its equivalent).

In yet a further aspect, the invention features a polypeptide (or analogues thereof) obtainable from a spider venom, that is an inhibitor of potassium channel

activity. The invention also features unique fragments of such a polypeptide. Such analogues, as defined above, are not themselves obtained from the venom but are derived by analysis of an isolated polypeptide (as exemplified herein) or can be obtained by screening other venoms.

The term "unique fragments" refers to portions that find no identical counterpart in known sequences as of the date of filing this application. These fragments can be identified easily by an analysis of polypeptide data bases existing as of the date of filing to detect counterparts.

By "pharmaceutically acceptable composition" is meant a therapeutically effective amount of a compound of the present invention in a pharmaceutically acceptable carrier, i.e., a formulation to which the compound can be added to dissolve or otherwise facilitate administration of the compound. Examples of pharmaceutically acceptable carriers include water, saline, and physiologically buffered saline. Such a pharmaceutical composition is provided in a suitable dose. Such compositions are generally those which are approved for use in treatment of a specified disorder by the FDA or its equivalent in non-U.S. countries.

By "disease or condition" is meant those diseases listed above and related diseases concerning cardiac or neural cells.

A further aspect within the scope of the invention is the use of such compounds as insecticidal agents. The compounds described herein are believed to possess significant insecticidal action, perhaps through block of a channel that is structurally similar to the transient outward K^+ channel of mammalian heart muscle. Spiders are known to produce venoms that contain a variety of toxins with potent insecticidal activities (Quistad, G.B., et al. "Insecticidal activity of spider (Araneae),

centipede (Chilopoda), scorpion (Scorpionidae), and snake (Serpentes) venoms", *Journal Economic Entomology* 85:33, 1992). These toxins have evolved in response to evolutionary pressures to produce effective toxins that effectively and rapidly kill or paralyse prey insects (Jackson, H. and P.N.R. Usherwood, "Spider toxins as tools for dissecting elements of excitary amino acid transmission", *Trends in Neurosciences* 11:278, 1988).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a chromatograph of *Heteropoda venatoria* venom (120 µl) fractioned on a Vydac C18 reversed-phase HPLC column (10 x 250 mm) equilibrated in 80% A/20% B. (A=0.1% aqueous trifluoroacetic acid (TFA (aq)), B=0.1% TFA in CH_3CN.) Diluted aliquots of whole venom in Solution A were injected at time 0. After 3 min. the solution was taken to 76%A/24%B over 1 min. At 5 min. elution was begun with a linear gradient from 76% A/24% B to 65% A/35% B over 44 min, with a flow rate of 3.5 ml/min. The absorbance of the eluant was monitored at 220 nm. The fractions (#1 - 8) are identified by number at the bottom of the absorbance trace. Fraction 5 contained Compound 4, Fraction 6 contained Compound 1 (SEQ ID NO. 1), and Fraction 7 contained Compound 2 (SEQ ID NO. 2).

Figs. 2 and 3 are chromatograms of peptide Compounds 1 and 2 fractionated on a cation-exchange column. Fig. 2: For peptide Compound 1 the column was developed with a linear gradient from 0-0.32 M NaCl in 50 mM sodium acetate, pH 4.0 in 32 min followed by a linear gradient from 0.32-1 M NaCl in 50 mM sodium acetate, pH

4.0 in 5 min. Fig. 3: For peptide Compound 2 the column was developed with a linear gradient from 0-0.3 M NaCl in 50 mM sodium acetate, pH 4.0 in 3 min followed by a linear gradient from 0.3-1 M NaCl in 50 mM sodium acetate, pH 4.0 in 35 min. Elution was at 1 ml/min and the effluent was monitored at 280 nm. Fractions were collected as noted on the chromatogram. (A quite similar chromatogram was obtained for a comparable purification from which Compound 4 was characterized.)

Fig. 4 is a chromatogram of *Olios fasciculatus* venom (108 µl) fractionated on a Vydac C-18 reversed phase column (300 Å, 10 x 250 mm). Five minutes after injection of the sample, the column was developed with a linear gradient from 20-45% acetonitrile/0.1% TFA in 75 minutes. At 50 minutes, the column was taken to 100% acetonitrile/0.1% TFA over 7 min. The flow rate was 3.0 ml/min and the effluent was monitored at 220 nm. Fractions were collected as noted on the chromatogram.

Fig. 5 is a chromatogram of peptide Compound 3 purified by cation-exchange chromatography. Five minutes after injection of the sample, the column was developed with a linear gradient from 0.25-1 M NaCl in 50 mM sodium acetate buffer, pH 4.0 in 75 min. Elution was at 1 ml/minute and the effluent was monitored at 280 nm. Fractions were collected as noted on the chromatogram.

Figs. 6 and 7 are graphs showing the fractional voltage dependent block of the potassium channel Kv4.2 expressed in *Xenopus* oocytes. Fig. 6 shows the block by Compound 2 (SEQ ID NO.2) and Compound 4 (SEQ ID NO. 4), while Fig. 7 shows the block by Compound 1 (SEQ ID NO. 1). The relationship between fractional block of current and test potential was determined using 100 nM for Compounds 1 and 4, and 67 nM of Compound 2 (n=4 for each compound).

The following is a detailed description of methods and tests by which useful compounds of this invention can be utilized for treatment of, *e.g.*, cardiac arrhythmias and disorders of memory and learning. An important method is the means by which compounds, both synthetic and natural products, can be rapidly screened with radioligand binding techniques (or their equivalent) to identify those which modify binding at the site on the transient outward K^+ channel bound by one of Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), Compound 3 (SEQ ID NO. 3), or Compound 4 (SEQ ID NO. 4). Screening for K^+ channel blockers from spider venom can be performed in a similar manner. In addition, useful derivatives or analogues or muteins of such polypeptides can be readily screened using this methodology.

Additional testing can include whole-cell recording of ionic currents in cardiac and neural cells to confirm that agents which compete with radiolabeled Compound 1, Compound 2, Compound 3, or Compound 4 binding act as specific active agents of transient outward K^+ currents, and are without significant effect on other channel types.

The desired properties of agents identified by means outlined in this invention include:

1) Specific and potent block of a potassium channel, *e.g.*, cardiac or neural transient outward K^+ channels. Specific block implies that the agent will not have demonstrable effects on other ionic channels or receptors at concentrations *in vitro* that block I_{to} or I_A at dosages that prove to be of therapeutic utility in the treatment of cardiac arrhythmias or disorders of learning and memory, or the other diseases listed above.

2) Lack of significant side effects at therapeutic doses, including excessive prolongation of QT interval of the electrocardiogram, bradycardia, hyperexcitability or seizures.

Isolation of Spider Venom Toxins

The following is a non-limiting example of methods by which inhibitory spider toxins of this invention may be isolated. Those in the art will recognize that equivalent methods can be used to isolate and identify other polypeptides (or their equivalent) having useful activity in this invention. Equivalent compounds are those referred to herein as analogues, muteins and derivatives which can be identified by methods described herein as having useful activity on one or more K^+ channels. Those in the art will recognize that once one useful K^+ channel active agent is identified and sequenced it can be chemically synthesized in totality or as fragments, and modifications in the sequence made, as described below. In addition, such fragments or the polypeptide itself may be used to screen for other such active agents which are equivalent in their activity to the original polypeptide.

Venom is obtained from the spiders *Heteropoda venatoria* and *Olios fasciculatus* through the process of milking by electrical stimulation according to standard methods well known to those skilled in the art. It is preferred that the method employed is one which safeguards against contamination of the whole venom by abdominal regurgitant or hemolymph. Such methods are well known to those skilled in the art. The whole venom so obtained is stored in a frozen state at about -78°C until used for purification as described below. Purification of the constituents from the whole venom is accomplished by reversed-phase high performance liquid chromatography (HPLC) on a variety of preparative and semi-preparative columns such as C-4 and C-18 Vydac columns (Rainin Instrument Co. Inc., Mack Road, Woburn, Massachusetts 01801). Peak detection is carried out monochromatically at 220 nm. Further analysis of the fractions can be

accomplished with, for example, polychrome UV data collected with a Waters 990 diode array detector (Millipore Corporation, Waters Chromatography Division, 34 Maple Street, Milford, Massachusetts 01757). The fractions from the columns are collected by known methods such as through the use of a fraction collector and an ISCO 2159 peak detector (ISCO, 4700 Superior, Lincoln, Nebraska, 68504). The fractions are collected in appropriately sized vessels such as sterile polyethylene laboratory ware. Concentration of the fractions is then accomplished by lyophilization from the eluant followed by lyophilization from water. Purity of the resulting constituent fractions then can be determined by chromatographic analysis using a different type of column than the system used in the final purification of the fractions.

Peptide Sequencing

The polypeptides of the invention, e.g., identified as described herein, can be sequenced according to known methods. A general strategy for determining the primary structure includes, for example, the following steps: 1) Reduction and S-pyridylation of disulfide-bridged cysteine residues to enhance substrate susceptibility to enzymatic attack; 2) Controlled cleavage of the peptide through single or multi-step enzymatic digestion; 3) Isolation and purification of peptide fragments via reversed-phase high performance liquid chromatography (HPLC); 4) Characterization of peptide fragments through N-terminal sequencing and ion-spray mass spectrometry.

S-pyridylethylation of cysteine residues of the polypeptides under study can be performed, for example, in solution followed by amino acid sequencing of the polypeptides. One such procedure for S-pyridylethylation can be accomplished as described below.

About 1 to 10 μg of polypeptide is dissolved or

diluted in up to 50 µl of a buffer prepared by mixing 1 part TrisHCl, pH 8.5, containing 4 mM EDTA and 3 parts 8M guanidine·HCl. 2.5 µl of 10% aqueous 2-mercaptoethanol is added and the mixture is incubated at room temperature in the dark under argon for two hours. After incubation, 2 µl of 4-vinylpyridine (fresh reagent stored under argon at - 20°C) is added and the mixture is incubated for another two hours at room temperature in the dark under argon. The mixture is then desalted, preferably by chromatography on a short reversed-phase column. The recovered alkylated polypeptide is then sequenced according to known methods.

Alternatively, the polypeptide can be sequenced after *in situ* reduction and S-pyridylethylation as described in Kruft et al., *Anal. Biochem.* 193:306 (1991).

Given the benefits of the disclosure herein with respect to the peptides Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), and Compound 4 (SEQ ID NO. 4) from the venom of *Heteropoda venatoria*, and peptide Compound 3 (SEQ ID NO. 3) of *Olios fasciculatus*, it is now possible to obtain other peptides by methods other than through isolation/purification from whole venom. The polypeptides of this invention can be produced using recombinant DNA techniques through the cloning of a coding sequence for said polypeptides or portions thereof. For example, hybridization probes which take advantage of the now known amino acid sequence information of said polypeptides can be employed according to methods well known to those skilled in the art to clone a coding sequence for the entire polypeptide. A combination of recombinant DNA techniques and *in vitro* protein synthesis can also be employed to produce the polypeptides of this invention. Such *in vitro* protein synthesis methods include, but are not limited to, use of an ABI 430A solid phase peptide synthesizer (Applied Biosystems, Inc., 850

Lincoln Center Drive, Foster City, California 94404) employing standard Merrifield chemistry or other solid phase chemistries well known to those skilled in the art.

Equivalent Peptides

It is well known in the art that certain amino acid substitutions can be made in polypeptides which do not affect, or do not substantially affect, the function of said polypeptides. The exact substitutions which are possible vary from polypeptide to polypeptide. Determination of permissible substitutions is accomplished according to procedures well known to those skilled in the art. Thus, all polypeptides having substantially the same amino acid sequence and substantially the same K^+ channel blocking activity are within the scope of this invention.

Biological Activity

The polypeptides or fragments thereof are useful in the treatment of cardiac arrhythmias or in disorders of memory and learning such as Alzheimer's disease and those other diseases noted above. When used for such indications, the peptides and fragments are formulated according to standard formulation methods known in the art, such as those disclosed in Remington's Pharmaceutical Sciences (latest edition, Mack Publishing Company, Easton, PA). The nature of the formulation will depend on the route of administration and the dosage required. Optimization of the dosage for a particular indication can be accomplished using standard optimization techniques as is generally practiced for peptide medicaments.

In general, administration by injection is preferred, either intravenous, intramuscular, subcutaneous or intraperitoneal. For injection, the peptide or fragments are formulated in liquid medium, such as Ringer's solution, Hank's solution, or other forms of physiological

saline. Formulations may also involve lyophilized preparations which can be reconstituted for administration. Alternative means of providing the active compounds of the invention to the subject include transmucosal and transdermal administration, wherein the formulation includes a permeation enhancer, such as a detergent, as well as additional excipients. Properly formulated, oral administration is also within the scope of the invention.

Types of Transient Outward Potassium Channels: Kv1.4, Kv4.2, and Kv4.3

Two different types of I_{to} potassium channels have been cloned from cardiac tissue. These are Kv1.4 (*Shaker* subfamily) and Kv4.2 (*Shal* subfamily), which were cloned from heart cDNA libraries, their mRNAs detected in Northern blots of heart tissue, and found to have properties similar to voltage-dependent cardiac I_{to} when studied in heterologous expression systems. (Roberds and Tankun, "Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart", *Proc. Natl. Acad. Sci. USA* 88:1793-1802, 1991; Blair et al., "Functional characterization of RK5, a voltage-gated K^+ channel cloned from the rat cardiovascular system", *FEBS Letters* 295:211-213, 1991; Po et al., "Functional expression of an inactivating potassium channel cloned from human heart", *Circ. Res.* 71:732-736, 1992.) However, Kv4.2 but not Kv1.4 was detectable in isolated rat myocytes using immunohistochemical techniques. This suggests that Kv4.2 subunits form functional I_{to} in rat heart muscle, and that Kv1.4 forms I_{to} channels in other tissues (*e.g.*, neural or smooth muscle) within the heart. It is likely that similar differences in distribution will be observed in other tissues.

While the presence of Kv4.2 was demonstrated in rat myocytes, recent results suggest that the highly

homologous Kv4.3 potassium channel is present in human heart tissue, while Kv4.2 appears to be present in human brain. (Dixon et al., "The Role of the Kv4.3 Channel in Cardiac Myocyte Function" , *Biophys, J.* 70:A307, 1996.)

Screening Assay Using Peptides Compounds 1, 2, 3, and 4

The peptides and biologically active fragments thereof are useful in screening assays to assess the ability of small molecules or other candidate drugs to inhibit the binding of Compounds 1, 2, 3, or 4 (SEQ ID NO. 1, 2, 3, and 4) to cardiac or neural transient outward K^+ channels. Described herein below is a suitable assay for competitive binding in which the Compound 1, 2, 3 or 4 of the invention are useful. For use in this assay, generally, the polypeptides Compound 1, 2, 3, or 4 (or an active fragment) is supplied in radiolabeled form and the ability of the candidate compound to compete with radiolabeled Compound 1, 2, 3, or 4 for binding to the potassium channel is assessed.

The following examples are intended to illustrate but not to limit the invention.

Example 1: *Heteropoda venatoria* Venom Reversed-Phase HPLC Fractionation

Approximately 900 µl of *Heteropoda venatoria* venom was fractionated by diluting 75-120 µl aliquots of the crude venom to 1 ml with A and applying the diluted fractions to a Vydac C18 column (10 x 250 mm) equilibrated in 20% B. (A=0.1% TFA (aq); B=0.1% TFA in CH_3CN). After 3 min, the gradient was changed to 24% B over 1 min and at 5 min, a linear gradient from 24% to 35% B over 44 min was begun. The flow rate was 3.5 ml/min and the effluent was detected at 220 nm (Fig. 1). The following fractions were collected: fraction 1 (peaks eluting between 5 and 16

minutes), fraction 2 (peaks eluting between 16 and 19 minutes), fraction 3 (peaks eluting between 19 and 23.5 minutes), fraction 4 (peaks eluting between 23.5 and 26.5 minutes), fraction 5 (peaks eluting between 26.5 and 29.5 minutes), fraction 6 (peaks eluting between 29.5 and 33 minutes), fraction 7 (peaks eluting between 33 and 37 minutes), fraction 8 (peaks eluting between 37 and 39 minutes), and the end fraction (peaks eluting between 39 and 46 minutes). At 39 min, after the majority of the venom components had eluted, the column was taken to 50% B over 3 min. When no further peaks eluted (~7 minutes) the column was returned to 20% B over 4 min and equilibrated for the next chromatography. Like fractions from the 8 chromatographic runs were combined and lyophilized. The major peaks of fractions 5, 6, and 7 correspond to Compounds 4, 1, and 2, respectively described in Examples 2, 4, and 7 below.

Example 2: *Heteropoda* Peptide Compound 1 Sequence Determination

Crude *Heteropoda venatoria* venom (~50 µl) was applied to a reversed phase HPLC column (Vydac, C-18 300 A, 22 x 250 nm) and was operated using a biphasic linear gradient program from 80% A and 20% B to 65% A and 35% B over 60 minutes (A = 0.1% trifluoroacetic acid (TFA), B = acetonitrile) with detection at 220 nm and a flow rate of 15 ml/min. The desired fraction was collected from 43 to 44 min. Pooled fractions from individual runs were concentrated by lyophilization.

The structure of peptide Compound 1 was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral

analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

A pyridylethylated derivative of Compound 1 suitable for N-terminal sequencing was generated *in situ* according to the method of Kruft et al., *Anal. Biochem.* 193:306 (1991).

The data taken together affirm the structure of peptide Compound 1 as shown below.

SEQ. ID. NO. 1:

```
Asp Asp Cys Gly Lys Leu Phe Ser Gly Cys Asp Thr Asn Ala
1               5                  10
Asp Cys Cys Glu Gly Tyr Val Cys Arg Leu Trp Cys Lys Leu
15                  20                  25
Asp Trp
     30
```

30 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 3412.86 (amide).
Observed mass = 3412.70 (ion spray m.s.).
Estimated pI = 3.76.

Example 3: *Heteropoda* Peptide Compound 1 Ion-Exchange Purification

Peptide Compound 1 (SEQ ID NO. 1) was also further purified by cation-exchange chromatography on a HEMA-IEC BIO SB column (10 μm, 4.6 x 150 cm; Alltech Associates, Deerfield, IL 60015). The lyophilized material containing peptide Compound 1 from the reversed-phase chromatography was dissolved in three ml of 50 mM sodium acetate, pH 4.0 and chromatographed in three equal portions as follows. One ml was loaded onto the HEMA-IEC BIO SB column equilibrated in 50 mM sodium acetate, pH 4.0. After 5 min, the column was developed with a linear gradient from 0-0.32 M NaCl in 50 mM sodium acetate, pH 4.0 in 32 min followed by a linear gradient from 0.32-1 M NaCl in 50 mM sodium acetate, pH 4.0 in 5 min (Fig. 2). After 10 min, the column was returned to the starting conditions in 5 min and equilibrated for subsequent chromatographies. Elution was at 1 ml/min and the

effluent was monitored at 280 nm. Fractions were collected as noted on the chromatogram. The remaining two ml of crude Compound 1 was chromatographed as described above and like fractions from the three chromatographies were combined.

The major absorbance peak, which eluted from the cation-exchange column between 26.5 and 29 min, was desalted on a Vydac C-18 reversed-phase column (10 x 250 mm, 300 Å). The pooled fraction (~10 ml) was loaded onto the reversed-phase column equilibrated in 20% acetonitrile/0.1% TFA. After 10 min, the column was developed with a linear gradient from 20-35% acetonitrile/0.1% TFA in 30 min at a flow rate of 3.5 ml/min and the effluent was monitored at 220 nm. The fraction eluting between 35.5 and 38 min was lyophilized to give 641 µg of purified peptide Compound 1. The observed mass of this peptide was 3412.72 (electrospray ionization).

Example 4: *Heteropoda* Peptide Compound 2 Sequence Determination

Crude *Heteropoda venatoria* venom (~50 µl) was applied to a reversed-phase HPLC column (Vydac, C-18, 300 A, 22x250 mm) and was operated using a biphasic linear gradient program from 80% A and 20% B to 65% A and 35% B over 60 min (A = 0.1% trifluoroacetic acid (TFA), B = acetonitrile) with detection at 220 nm and a flow rate of 15 ml/min. The desired fraction was collected from 46 to 48.5 min. Pooled fractions from individual runs were concentrated by lyophilization.

The material from the fractionation above, derived from 50 µl of crude venom, was applied to a reversed-phase HPLC column (Vydac, C-18, 300 A, 22 x 250 mm) and was operated using an isocratic program of 75% A and 25% B + (A = 0.1% TFA, B = acetonitrile) with detec-

tion at 220 nm and a flow rate of 3.5 ml/min. The desired fraction was collected from 55 to 68 min. Pooled like fractions from individual runs were concentrated by lyophilization.

The structure of peptide Compound 2 was determined and verified by the following methods. PTC amino acid analysis was carried out on 1-10 nmols in triplicate using the Waters Pico-Tag system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and reduced/pyridylethylated peptide. Mass spectral analysis was obtained from a SCI-EX API III ion spray mass spectrometer.

A pyridylethylated derivative of Compound 2 suitable for N-terminal sequencing was generated *in situ* according to the method of Kruft et al., *Anal. Biochem.* 193:306-309, 1991.

The data taken together affirm the structure of peptide Compound 2 as shown below.

SEQ. ID. NO. 2:

```
Glu Cys Gly Thr Leu Phe Ser Gly Cys Ser Thr His Ala Asp
1               5                  10
Cys Cys Glu Gly Phe Ile Cys Lys Leu Trp Cys Arg Tyr Glu
15                 20                  25
Arg Thr Trp
    30
```

31 residues, 6 cysteines, 3 disulfide bonds.
Calculated mass = 3599.05 (amide).
Observed mass = 3599.38 (ion spray m.s.)
Estimated pI = 5.41.

Example 5: *Heteropoda* Peptide Compound 2 Ion-Exchange Purification

Peptide Compound 2 was also purified by cation-exchange chromatography on a HEMA-IEC BIO SB column (10 μm, 4.6 x 150 cm). The lyophilized material containing peptide Compound 2 from the initial reversed-phase chromatography was dissolved in three ml of 50 mM sodium

acetate, pH 4.0 and chromatographed in three equal portions as follows. One ml was loaded onto the HEMA-IEC BIO SB column equilibrated in 50 mM sodium acetate, pH 4.0. After 5 min, the column was developed with a linear gradient from 0-0.3 M NaCl in 50 mM sodium acetate, pH 4.0 in 3 min followed by a linear gradient from 0.3-1 M NaCl in 50 mM sodium acetate, pH 4.0 in 35 min (Fig. 3). After 5 min the column was returned to the starting conditions over 10 min and equilibrated for subsequent chromatographies. Elution was at 1 ml/min and the effluent was monitored at 280 nm. Fractions were collected as noted on the chromatogram. The remaining two ml of crude Compound 2 was chromatographed as described above and like fractions from the three chromatographies were combined.

The major absorbance peak, which eluted from the cation-exchange column between 30 and 34 min, was desalted in two portions on a Vydac C-18 reversed-phase column (10 x 250 mm, 300 Å). The column was equilibrated in 25% acetonitrile/0.1% TFA and eluted with the starting solvent for 10 min, followed by a linear gradient from 25-35% acetonitrile/0.1% TFA in 20 min at a flow rate of 3.5 ml/min. The effluent was monitored at 220 nm and peptide Compound 2 eluted as a single peak from 26.5 to 29 min. The remaining pool from the cation-exchange column was then desalted and like fractions were combined. This pool was lyophilized to give 1.88 mg of purified peptide Compound 2. The observed mass of this peptide was 3599.52 (electrospray ionization).

Example 6: *Heteropoda* Peptide Compound 4 Ion-Exchange Purification

Peptide Compound 4 was also purified by cation-exchange chromatography on a HEMA-IEC BIO SB column (10 µm, 4.6 x 150 cm). The lyophilized material containing

peptide Compound 4 from the initial reversed-phase chromatography was dissolved in 50 mM sodium acetate, pH 4.0 and chromatographed as follows. An aliquot was loaded onto a HEMA-IEC BIO SB column equilibrated in 50 mM sodium acetate, pH 4.0. The column was developed with a linear gradient from 0.1-1.0 M NaCl in 50 mM sodium acetate, pH 4.0 over 45 min. Elution was at 1 ml/min and the effluent was monitored at 280 nm.

The major absorbance peak was desalted on a Vydac C-18 reversed-phase column (10 x 250 mm, 300 Å). The column was equilibrated in 0.1% TFA, developed with a 10 min linear gradient from 0 to 25 % Solution B, and eluted with a linear gradient from 25-35% Solution B in 20 min at a flow rate of 3.5 ml/min. The effluent was monitored at 220 nm and peptide Compound 4 eluted as a single peak from 24 to 25.5 min. The observed mass of this peptide was 3910.57 (ion spray mass spectrometer, see Example 7 below).

Example 7: *Heteropoda* Peptide Compound 4 Sequence Determination

Portions of the cation exchange purified Compound 4 prepared as described above were used for N-terminal sequencing and mass spectral analysis.

The mass of Compound 4 was determined using a triple quadrupole mass spectrometer with an ionspray interface (Perkin-Elmer SCIEX API III system). The sample was dissolved in 10% acetic acid and the sample solution (0.2 mg/mL) was delivered at a flow rate of 1.0 µl/min to a sprayer by a syringe infusion pump. The molecular mass of the sample was determined with the first quadrupole, which was calibrated with the ammonium adduct ions of poly(propylene glycols).

PTC amino acid analysis was performed on 1-10 nmols of the peptide in triplicate using a Waters Pico-Tag

system. N-terminal sequencing was carried out on a pulse-liquid sequenator (ABI) on both native and S-pyridylethylated derivatives. S-pyridylethylated peptides were generated *in situ* according to the method of Kruft et al., *Anal. Biochem.* 193:306-309, 1991.

Taken together, the data affirms the structure of Compound 4 as shown below.

SEQ ID NO. 4:

```
Asp Cys Gly Thr Ile Trp His Tyr Cys Gly Thr Asp Glu Ser
1               5                  10
Glu Cys Cys Glu Gly Trp Lys Cys Ser Arg Glu Leu Cys Lys
15                 20                  25
Tyr Val Ile Asp Trp
     30
```

33 residues, 6 cysteines
Observed mass = 3910.57 (ion spray m.s.)

Example 8: Fractionation of *Olios fasciculatus* venom

Approximately 108 µl of *Olios fasciculatus* venom was fractionated by diluting the whole venom with 1.5 ml of 20% acetonitrile/0.1% TFA and loading the sample on to a Vydac C-18 column (300 Å, 10 X 250 mm) equilibrated in the same buffer. Five minutes after injection of the sample, the column was developed with a linear gradient from 20-45% acetonitrile/0.1% TFA in 75 min (Fig. 4). At 50 min, after the majority of the venom components had eluted, the column was taken to 100% acetonitrile/0.1% TFA over 7 min. The flow rate was 3.0 ml/min and the effluent was monitored at 220 nm. Fractions were collected as noted on the chromatogram. The fraction (#21) containing peptide Compound 3, which eluted between 40 and 42 min, was lyophilized and the residue dissolved in 2 ml of 50 mM sodium acetate, 0.25 M NaCl, pH 4.0.

Example 9: *Olios fasciculatus* Compound 3 Ion-Exchange Purification

Peptide Compound 3 was further purified by cation exchange chromatography on a HEMA-IEC BIO SB column (10 μm, 4.6 x 150 cm, from Alltech Associates, Deerfield, IL 60015). The solution containing peptide Compound 3 (1.5 ml) from the reversed-phase chromatography was loaded onto the HEMA-IEC BIO SB column equilibrated in the same buffer. After 5 minutes, the column was developed with a linear gradient from 0.25-1 M NaCl in 50 mM sodium acetate buffer, pH 4.0 in 75 min (Fig 5). Elution was at 1 ml/min and the effluent was monitored at 280 nm. Fractions were collected as noted on the chromatogram.

The major peak of material (fraction #4), which eluted from the cation-exchange column between 27 and 32 min, was desalted on a Vydac C-18 reversed-phase column (10 x 250 mm, 300 Å). The fraction (~4.5 ml) was loaded onto the reversed-phase column equilibrated in 0.1%TFA. After 3 min, the column was developed with a linear gradient from 0-15% *iso*-propanol/0.1% TFA in 3 min followed by a linear gradient from 15-30% *iso*-propanol/0.1% TFA in 30 min and from 30-50% *iso*-propanol/0.1% TFA in 5 min. Elution was at 1.0 ml/min and the effluent was monitored at 220 nm. The fraction eluting between 43 and 45 min was lyophilized to give 70 μg of purified peptide Compound 3. The observed mass of this peptide was 3786.64 (electrospray ionization).

N-terminal sequence analysis was obtained for reduced, derivatized peptide Compound 3. The sequence is as follows:

SEQ. ID. NO. 3:

```
Asp Asp Cys Ala Gly Trp Met Glu Ser Cys Ser Ser Lys
 1               5                  10
Pro Cys Cys Ala Gly Arg Lys Cys Phe Ser Glu Trp Tyr
    15                  20                  25
Cys Lys Leu Val Val Asp Gln Asn
            30
```

34 residues, 6 cysteines, 3 disulfide bonds.
Observed mass = 3786.64 (ion spray m.s.)

There is low confidence in the identity of amino acids 33 and 34.

Example 10: K+ Channel Blocking Activity of *Heteropodavenatoria* and *Olios fasciculatus* Peptide Fractions, and Compounds 1, 2, 3, and 4 in Rat Ventricular Myocytes

The ability of the peptide fractions and Compounds 1, 2 and 3 of this invention to block transient outward K^+ channels is demonstrated by the following procedure.

Rat ventricular myocytes were isolated according to the procedure described previously (Kamp et al., "Voltage- and Use-dependent Modulation of Cardiac Calcium Channels by the Dihydropyridine (+)-202-791", *Circ. Res.* 64:338, 1989). The method involves retrograde perfusion of an excised rat heart with a solution containing collagenase and protease to enzymatically digest the entire heart so as to isolate individual cardiac myocytes suitable for use in standard voltage clamp experiments. Whole-cell currents are recorded from isolated myocytes using the voltage clamp techniques described in detail elsewhere (Hamill et al., "Improved Patch Clamp Techniques for High-resolution Current Recording from Cells and Cell-free Membrane Patches", *Pflugers Arch.* 391:85, 1981). In other tests, quite similar results were obtained using a closely related method as described in Giles and Shibata, "Voltage clamp of bull-frog cardiac pacemaker cells: A quantitative analysis of potassium currents", *J. Physiol. (Lond.)* 368:265-292 (1985). Cells are placed in a 0.5 ml recording chamber and bathed in a buffered solution of the following composition (in mM): NaCl, 132; $MgCl_2$, 1.2; $CaCl_2$, 1.8; KCl, 4; HEPES, 10; glucose, 10; pH

= 7.4. In most experiments in which K^+ currents were recorded, Ca^{2+} current was blocked by omission of $CaCl_2$ and addition of 1 mM Co^{2+} to this solution. The myocytes are voltage clamped using a commercially available patch clamp amplifier (Axon Instruments Axopatch 1D), and data acquisition and analysis is performed using a personal computer. Cells were clamped at a potential of -60 mV. Test potentials (500 msec duration) were applied to potentials ranging from -40 to +30 mV. Using these techniques several K^+ currents can be recorded in these cells, including an inward rectifier K^+ current; a rapidly activating, non-inactivating delayed rectifier K^+ current; and a voltage-dependent transient outward K^+ current (I_{to}). The dried fraction residues of venom fractions 1-8 and the end fraction prepared as described in Example 1 were each dissolved in 1 ml of water. A 10 µl sample of each was then diluted with 3 ml of the buffered solution to test for effects on cardiac K^+ currents. Under these conditions, peptide fractions 2-9 blocked I_{to} in a voltage-dependent manner. Block was complete at a test potential of -10 mV, with block reduced to 30 to 70% of control values at a test potential of +30 mV. The predominant peptides of fractions 6 (Compound 1 (SEQ ID NO. 1)) and 7 (Compound 2) were isolated and purified as described in Examples 3 and 5 above. Compound 1 blocked I_{to} in a voltage-dependent manner, with greater block occuring at less depolarized test potentials. This was quantified by determining the concentration required to inhibit I_{to} by 50% (IC_{50}) and the maximum block of this current at three different test potentials. The maximum block of I_{to} at test potentials of -10 mV, +20 mV and +50 mV was 100%, 79% and 69%, respectively. The IC_{50} for block of I_{to} was 16 nM at -10 mV, 35 nM at +20 mV, and 138 nM at +50 mV (n = 4-6). Consistent with block of I_{to}, Compound 1 (30 nM) prolonged action potential duration, measured at 90%

repolarization, of isolated rat ventricular myocytes by 34±5% (n=5). Selective prolongation of action potential duration represents class III antiarrhythmic activity (Vaughan Williams, "Delayed ventricular repolarization as an antiarrhythmic principle", *Eur. Heart J.* 6:145, 1985). The effects of Compound 1 or 2 on other cardiac currents was determined to assess their specificity. At concentrations of 0.2 - 1.0 μM, Compound 1 or Compound 2 did not affect the following cardiac currents as measured using standard whole cell-voltage clamp techniques:

-ultrarapid delayed rectifier K^+ current (I_{Kur}) in rat ventricular myocytes

-slow delayed rectifier K^+ current (I_{Ks}) in guinea pig ventricular myocytes

-inward rectfier K^+ current (I_{K1}) in rat and guinea pig ventricular myocytes

-sodium current (I_{Na}) in rat ventricular myocytes

-L-type Ca^{2+} current (I_{Ca-L}) in rat and guinea pig ventricular myocytes

In an isolated human ventricular myocyte, Compound 1 (0.2 μM) also blocked I_{to} but not I_{Kur}, similar to the findings in rat ventricular myocytes. I_{to} in human myocytes is believed to be be due to Kv4.3 channels.

Compound 3 had similar activity on rat ventricular myocytes, blocking I_{to} completely at test potentials < 0 mV at 1 μM, while having no effect on either delayed rectifier or inward rectifier K^+ currents. The effects of Compound 1 and Compound 2 (1 μM) were substantially reversed upon washout of the toxins.

Compound 1 or 2 was also tested for activity on a number of other K^+ channels recorded from isolated non-cardiac cells. At 0.2 - 1.0 μM, Compound 1 or 2 had no effect on:

-rapid delayed rectifier K^+ currents (I_K) of rat neural cells (Purkinje neurons, cerebellar granule cells,

hippocampal pyramidal cells, sympathetic ganglion cells), GH, pituitary cells, or rabbit osteoclasts.

-transient outward current (I_A) of rat cerebellar granule cells or sympathetic ganglion cells.

-a cloned channel (Kv1.4) expressed in *Xenopus* oocytes.

Thus, Compounds 1 and 2 were shown to be quite specific for one type of channel (a voltage-activated, transient outward K^+ current) in cardiac myocytes. The only other toxin reported to inhibit a transient outward K^+ current (in neural cells) is dendrotoxin (Haliwell et al., "Central action of dendrotoxin: Selective reduction of a transient K conductance in hippocampus and binding to localized acceptors", *Proc. Natl. Acad. Sci.* USA 83:493, 1986). However, we have shown that dendrotoxin (2 μM) has no effect on rat cardiac I_{to}. Therefore, Compounds 1, 2 and 3 are the first toxins described that specifically block cardiac I_{to}.

Example 11: Neural effects of Compound 1, 2, and 4

The ability of Compounds 1, 2, and 4 (SEQ ID NO. 1, 2, and 4) of this invention to affect neural activity is demonstrated by their electrophysiological effects on hippocampal slices.

Male Sprague-Dawley rats (100-200 g) were sacrificed by decapitation. The brain was removed from the cranium, immediately placed in cold (4-6°C), oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) consisting of (in mM) NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; glucose, 11, and slices of hippocampus were prepared as described previously (Mueller et al., "Arylamine spider toxins antagonize NMDA receptor-mediated synaptic transmission in rat hippocampal slices", *Synapse* 9:244, 1991). Slices were maintained in a reservoir of 200 ml of oxygenated aCSF at room temperature. Following a 1 hr recovery period, a single

slice was transferred to a small volume (~300 µl) recording chamber. Small platinum weights were placed on the slice to increase the stability of the recording. The slice was covered with aCSF and a superfusion system maintained the flow of fresh, oxygenated aCSF at 2 ml/min. The slice was held submerged in the flow of aCSF so that potential problems with access of drugs into the slice were minimized. The temperature in the recording chamber was held at 33°C for extracellular field potential recording. Bipolar concentric stimulating electrodes were placed under visual guidance in the stratum radiatum near the border of CA1-CA2. To evoke synaptic responses, monophasic 50 µsec pulses of 3-50 V were delivered to the slice every 30 sec while testing the response until potentials of maximal amplitude were obtained from a particular recording site. The voltage was then set so as to evoke a half-maximal response. Recording was done with 2-3 MW glass microelectrodes filled with 0.9% NaCl, which were also placed under visual guidance. Synaptic responses were recorded from the CA1 pyramidal cell layer (population spike) or from stratum radiatum (field excitatory postsynaptic potential (EPSP) and afferent volley (AV)), digitized, and entered into an IBM PC-based data acquisition and storage system. Toxins were made up in phosphate-buffered saline (PBS: NaCl, 140 mM; KCl, 2.5 mM; KH_2PO_4, 1.5 mM; Na_2HPO_4, 8.1 mM; pH 7.4) at 100-1000 times the desired final concentration, and then transferred to the reservoir syringe so as to achieve, via dilution and mixing, the desired final concentration. All drugs and toxins were applied by superfusion for 30 min, at which time the response amplitude had generally plateaued. All waveforms were digitized and stored on disk. Extracellularly recorded response amplitudes were averaged over a 5 min period just prior to drug application (control, pre-drug) and again over a 5 min

period following drug application (25-30 min post-drug).

Compound 1 produced a sustained increase in the population spike amplitude which did not recover during washout with fresh aCSF. The mean response to 500 nM Compound 1 was a 35 ± 9% increase (mean ± S.E.M., n = 5 slices). In two of these slices, the amplitude of the simultaneously recorded field EPSP was increased by an average of 16%, while the afferent volley was unchanged.

Similar results were obtained using purified Compound 2, which produced an increase in the population spike amplitude of 25 ± 7% (mean ± S.E.M., n = 9 slices) when applied at a final concentration of 1 μM. In two of these slices, the amplitude of the simultaneously recorded field EPSP was increased by an average of 12%, while the afferent volley was unchanged.

In another set of tests under very similar conditions, Compound 1 increased population spike amplitude by 35±9% (500nM, n=5) while Compound 2 increased population spike amplitude 22±7% (1 μM, n=6) (essentially the same as shown above). In contrast, Compound 4 suppressed population spike amplitude by 47±12% (1 μM, n=6). For these results, population spike amplitudes were averaged over a 5 min period just prior to drug application for the pre-drug control, and over a 5 min period following drug application for test values.

Taken together, these results demonstrate that Compounds 1 and 2 produce long-term increases in synaptic transmission at the Schaffer collateral-CA1 pyramidal cell synapse. These data cannot distinguish between a pre- and post-synaptic site of action, nor can they point clearly to a mechanism of action. Such increases in synaptic transmission could be due to blockade of voltage-sensitive potassium channels.

Many K^+ channel blocking agents can cause seizures when injected intravenously (i.v.), or when

administered by intracerebroventricular (i.c.v.) injection. For example, dendrotoxin causes convulsions and death in mice when injected i.c.v. at 0.008 μg/g, equivalent to about 0.24 μg/mouse (Schweitz, H. et al., "Purification and pharmacological characterization of peptide toxins from the black mamba (*Dendroaspis polylepis*) venom", *Toxicon* 28:847, 1990). In contrast, Compound 2 did not cause convulsions or seizures in audiogenic seizure-prone mice injected i.c.v. with 1 μg (n=3) or 2 μg (n=1) of Compound 2. After i.v. injection at doses of 10, 15 or 24 μg (n=1 each dose), Compound 2 caused a transient ataxia in mice, but convulsions were not observed.

Example 12: Other K^+ Channel Blocking Toxins

Compounds 1, 2, 3 and 4 represent the first reported examples of toxins isolated from spider venoms that block specific K^+ channels. Venoms from species of spiders other than *Heteropoda venatoria* and *Olios fasciculatus* may contain structurally unrelated toxins (peptides and nonpeptides) that potently block I_{to} or other types of K^+ channels in mammalian cells.

Several other toxins isolated from venoms of invertebrate and vertebrate venoms have been well characterized. For example, two toxins have been isolated from venom of the bee *Apis mellifera* that block K^+ channels. Apamin blocks a low conductance Ca^{2+}-activated K^+ channel, whereas MCD (mast cell degranulating) peptide blocks a non-inactivating delayed rectifier K^+ channel (Strong, "Potassium Channel Toxins", *Pharmac. Ther.* 46: 137, 1990). Other K^+ channel specific blocking toxins have been isolated from venoms produced by scorpions and snakes. For example, venom from the scorpion *Leiurus quinquestriatus* contains at least two toxins, charybdotoxin and leiurotoxin that block high conductance, and low

conductance Ca^{2+}-activated K^+ channels, respectively (Strong, "Potassium Channel Toxins", *Pharm. Ther.* 46:137, 1990). Toxins that block non-inactivating delayed rectifier K^+ channels of neurons have also been isolated from the venoms of mamba snakes (Harvey and Anderson, "Dendrotoxins: Snake Toxins That Block Potassium Channels and Facilitate Neurotransmitter Release", *Pharmac. Ther.* 31:33, 1985). Dendrotoxin from the green mamba snake (*Dendroaspis angusticeps*) and Toxin 1 from the black mamba (*D. polylepis*) both share considerable sequence homology with β-bungarotoxin, an inhibitory presynaptic neurotoxin isolated from venom of *Bungarus multicinctus* that also blocks the same type of K^+ channel (Moczydlowski et al., "An Emerging Pharmacology of Peptide Toxins Targeted Against Potassium Channels", *J. Membrane Biol.* 105:95, 1988). The above noted toxins are reported to have effects beyond block of non- inactivating delayed rectifier K^+ channels. For example, β-bungarotoxin also exhibits phospholipase A2 activity (Moczydlowski et al.) and dendrotoxin also blocks sodium current and a slow inactivating transient K^+ current in hippocampal neurons (Li and McArdle, "Dendrotoxin Inhibits Sodium and Transient Potassium Currents in Murine Hippocampal Neurons", *Biophys. J.* 64:A198, 1993).

The above-noted toxins have been useful in defining the role of specific K^+ channels in the physiology of normal cells and cells of diseased tissues. However, there are several K^+ channels known for which no highly specific and potent modulators have been discovered. This invention demonstrates that spider venoms represent an untapped source for the discovery of such novel channel ligands.

The existence of K^+ channel-specific toxins in spider venoms is examined by testing the effects of whole venoms, venom fractions separated by standard HPLC

methodology, and isolated toxins on K^+ currents measured using standard whole-cell voltage clamp recording techniques on isolated mammalian cardiac and neural cells as described in Example 10 above.

Example 13: Method for Screening Compounds that Bind to a Compound 1/Compound 2/Compound 3/Compound 4 Site on the Transient Outward K^+ Channel in Neural Tissue

Compound 1, 2, 3, or 4 (SEQ ID NO. 1, 2, 3, and 4) or related peptides are labeled with ^{125}I by procedures known in the art (lactoperoxidase, Bolton-Hunter, chloramine T, etc.). Candidate compounds acting at the Compound 1/Compound 2/ Compound 3/Compound 4 binding site are assessed by determining their ability to displace specific binding of [^{125}I]Compound 1, [^{125}I]Compound 2, [^{125}I]Compound 3, [^{125}I]Compound 4 or related peptides labeled with ^{125}I using techniques described below. The binding sites may be the same, different, or overlapping for each of Compounds 1, 2, 3, and 4.

The following assay can be utilized as a high throughput assay to screen product libraries (e.g., natural product libraries and compound files at major pharmaceutical companies) to identify new classes of compounds with activity at the Compound 1/Compound 2 /Compound 3/Compound 4 binding site on the I_{to} channel. These new classes of compounds are then utilized as chemical lead structures for a drug development program targeting the Compound 1/Compound 2/Compound 3/Compound 4 binding site on the neural I_{to} channel. The compounds identified by this assay offer a novel therapeutic approach to disorders of learning and memory such as Alzheimer's disease, and those other diseases listed above. It is important to demonstrate that a peptide retains its biological activity if it is to be used in a

quantitative binding assay. Iodinated (^{125}I) Compound 1, Compound 2, Compound 3, and Compound 4 retain their normal activity with regard to block of cardiac I_{to}. For example, ^{125}I-Compound 1 blocked I_{to} of rat ventricular myocytes in a voltage-dependent manner, with approximate IC_{50}'s of 25 nM at -10 mV, 70 nM at +20 mV, and 150 nM at +50 mV. The IC_{50} compares favorably with the IC_{50} for non-iodinated samples of Compound 1.

Rat brain membranes are prepared according to the method of Williams et al. ("Effects of Polyamines on the Binding of [^{3}H]MK-801 to the NMDA Receptor: Pharmacological Evidence for the Existence of a Polyamine Recognition Site", *Molec. Pharmacol.* 36:575, 1989) as follows: Male Sprague-Dawley rats (Simonsen Laboratories) weighing 100-200 g are sacrificed by decapitation. The brains from 20 rats (minus cerebellum and brainstem) are homogenized at 4°C with a glass/Teflon homogenizer in 300 ml 0.32 M sucrose containing 5 mM K-EDTA (pH 7.0). The homogenate is centrifuged for 10 minutes at 1,000 x *g* and the supernatant removed and centrifuged at 30,000 x *g* for 30 minutes. The resulting pellet is resuspended in 250 ml 5 mM K-EDTA (pH 7.0) stirred on ice for 15 minutes, and then centrifuged at 30,000 x *g* for 30 minutes. The pellet is resuspended in 90 ml 5 mM K-EDTA (pH 7.0), and 15-ml aliquots are layered over discontinuous sucrose gradients of 0.9 M and 1.2 M sucrose (10 ml each). The gradients are centrifuged at 95,000 x *g* for 90 minutes, and the synaptic plasma membrane (SPM) fraction at the 0.9 M/1.2 M sucrose interface collected. Membranes are washed by resuspension in 500 ml 5 mM K-EDTA (pH 7.0), incubated at 32°C for 30 minutes, and centrifuged at 100,000 x *g* for 30 minutes. The wash procedure, including the 30 minutes incubation, is repeated three times. The final pellet is resuspended in 60 ml 5 mM K-EDTA (pH 7.0) and stored in aliquots at -80°C. To perform a binding assay with

[^{125}I]Compound 1, 2 or 3, aliquots of synaptic plasma membranes (SPMs) are thawed, washed once by incubation at 32°C for 30 minutes, and centrifuged at 100,000 x *g* for 30 minutes. SPMs are resuspended in buffer A (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0). The [^{125}I]Compound 1, 2, 3, or 4 is added to this reaction mixture. Binding assays are carried out in polypropylene test tubes. The final incubation volume is 200 µl. Nonspecific binding is determined in the presence of 100 µM nonradioactive Compound 1, 2, 3, or 4. Triplicate samples are incubated at 32°C for 2 hours. Assays are terminated by the addition of 10 ml of ice-cold buffer A, followed by filtration over glass-fiber filters (Schleicher & Schuell No. 30). The filters are washed with another 10 ml of buffer A, and radioactivity is determined by gamma counting for ^{125}I.

In order to validate the above assay, the following experiments are also performed:

(a) The amount of nonspecific binding of the [^{125}I]Compound 1, 2, 3, or 4 to the filters is determined by passing 200 µl of buffer A containing 100 nM [^{125}I]Compound 1, 2, 3,or 4 through the glass-fiber filters. The filters are washed with another 10 ml of buffer A, and radioactivity bound to the filters is determined by scintillation counting. If a significant amount of nonspecific binding of the [^{125}I]Compound 1, 2, 3, or 4 occurs, then filters are prewashed with unlabeled Compound 1, 2, 3, or 4 to limit this binding. If high nonspecific binding remains a problem, assays will be terminated by centrifugation rather than by filtration, and the amount of radioactivity in the pellet will be determined by scintillation counting.

(b) A saturation curve is constructed by resuspending SPMs in buffer A. The assay buffer (200 µl) contains 75 µg of protein. Nine concentrations of

[^{125}I]Compound 1, 2, 3, or 4 are used, ranging from 10 nM to 100 μM in half-log units. A saturation curve is constructed from the data, and an apparent K_D value and B_{max} value determined by Scatchard analysis (Scatchard, "The Attractions of Proteins for Small Molecules and Ions", *Ann. N.Y. Acad. Sci.* 51:660, 1949). The cooperativity of binding of the [^{125}I]Compound 1, 2, 3, or 4 is determined by the construction of a Hill plot (Hill, "A New Mathematical Treatment of Changes of Ionic Concentrations in Muscle and Nerve Under the Action of Electric Currents, With a Theory to Their Mode of Excitation", *J. Physiol.* 40:190, 1910).

(c) The dependence of binding on protein (receptor) concentration is determined by resuspending SPMs in buffer A. The assay buffer (200 μl) contains a concentration of [^{125}I]Compound 1, 2, 3, or 4 equal to its K_D value and increasing concentrations of protein. The specific binding of [^{125}I]Compound 1, 2, 3, or 4 should be linearly related to the amount of protein (receptor) present.

(d) The time course of ligand-receptor binding is determined by resuspending SPMs in buffer A. The assay buffer (300 μl) contains a concentration of [^{125}I]Compound 1, 2, 3, or 4 equal to its K_D value and 100 μg of protein. Triplicate samples are incubated at 32°C for varying lengths of time; the time at which equilibrium is reached is determined, and this time point is routinely used in all subsequent assays.

(e) The pharmacology of the binding site can be analyzed by competition experiments. In such experiments, the concentration of [^{125}I]Compound 1, 2, 3, or 4 and the amount of protein are kept constant, while the concentration of test (competing) drug is varied. This assay allows for the determination of an IC_{50} and an apparent K_D for the competing drug (Cheng and Prusoff,

"Relationship Between the Inhibition Constant (K_i) and the Concentration of Inhibitor Which Causes 50 Percent Inhibition (IC_{50}) of an Enzymatic Reaction", *J. Biochem. Pharmacol.* 22:3099, 1973). The cooperativity of binding of the competing drug is determined by Hill plot analysis.

Specific binding of the [^{125}I]Compound 1, 2, 3, or 4 represents binding to a novel site on the I_{to} channel. As such, peptides related to Compound 1, 2, 3, or 4 should compete with the binding of [^{125}I]Compound 1, 2, 3, or 4 in a competitive fashion, and their potencies in this assay should correlate with their inhibitory potencies in a functional assay of I_{to} block (*e.g.*, inhibition of I_{to} in isolated neural or cardiac cells). Conversely, compounds which have activity at the other sites on the I_{to} channel should not displace [^{125}I]Compound 1, 2, 3, or 4 binding in a competitive manner. Rather, complex allosteric modulation of [^{125}I]Compound 1, 2, 3, or 4 binding, indicative of noncompetitive interactions, might be expected to occur.

(f) Studies to estimate the dissociation kinetics are performed by measuring the binding of [^{125}I]Compound 1, 2, 3, or 4 after it is allowed to come to equilibrium (see (d) above), and a large excess of nonradioactive competing drug is added to the reaction mixture. Binding of the [^{125}I]Compound 1, 2, 3, or 4 is then assayed at various time intervals. With this assay, the association and dissociation rates of binding of the [^{125}I]Compound 1, 2, 3, or 4 are determined (Titeler, "*Multiple Dopamine Receptors*: Receptor Binding Studies in Dopamine Pharmacology", Marcel Dekker, Inc., New York, 1983). Additional experiments involve varying the reaction temperature (20°C to 37°C) in order to understand the temperature dependence of these parameters.

Example 14: Method for Screening Compounds that Bind to

a Compound 1/Compound 2/Compound 3/Compound 4 Site on the Transient Outward K^+ Channel in Cardiac Tissue

Compound 1, Compound 2, Compound 3, Compound 4 (SEQ ID NO. 1, 2, 3, and 4) and related peptides are labeled with ^{125}I by procedures known in the art (lactoperoxidase, Bolton-Hunter, chloramine T, etc.). Candidate compounds acting at the Compound 1/Compound 2/Compound 3/Compound 4 binding site are assessed by determining their ability to displace specific binding of [^{125}I]Compound 1, [^{125}I]Compound 2, [^{125}I]Compound 3, [^{125}I]Compound 4 or related peptides labeled with ^{125}I or other detectable label using techniques known to those skilled in the art, for example, as described below.

The following assay can be utilized as a high throughput assay to screen product libraries (*e.g.*, natural product libraries and compound files at major pharmaceutical companies) to identify new classes of compounds with activity at the Compound 1/Compound 2/Compound 3/Compound 4 binding site on the cardiac I_{to} channel. These new classes of compounds are then utilized as chemical lead structures for a drug development program targeting the Compound 1/Compound 2 binding/Compound 3/Compound 4 site on the cardiac I_{to} channel. The compounds identified by this assay offer a novel therapeutic approach to the treatment of reentrant supraventricular and ventricular cardiac arrhythmias.

Cardiac sarcolemmal vesicles are prepared according to the method of Doyle et al. ("Saxitoxin binding and "Fast" Sodium Channel Inhibition in Sheep Heart Plasma Membrane", *Am. J. Physiol.* 249:H328, 1985) and Jones and Besch ("Isolation of Canine Cardiac Sarcolemmal Vesicles", *Methods in Pharmacology* 5:1, 1984), as modified by Kamp and Miller ("Voltage-dependent Nitrendipine Binding to Cardiac Sarcolemmal Vesicles",

Mol. Pharmacol. 32:278, 1987). Cardiac sarcolemmal vesicles are prepared from fresh bovine or other suitable mammalian heart tissue at 0-4°C. The heart is cut into 1 cm^3 pieces, and converted into a paste with a meat grinder. The paste was homogenized in 4-times its volume of 0.75 M choline Cl buffered to pH 7.4 with 30 mM *N*-2-hydroxyethyl-piperazine-*N'*-2- ethanesulfonic acid (HEPES)-15 mM Tris. The homogenization was carried out twice for 30 seconds in 300 ml polypropylene centrifuge jars with a Tekmar T135 shaft. This and all other buffers include the proteinase inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, and 1 mM dithiothreitol. The resultant homogenate is centrifuged for 20 minutes at 27,000 x *g* in the GSA rotor of a Sorvall centrifuge. The supernatant is discarded, and the pellet is resuspended in 10 mM HEPES-5 mM Tris, pH 7.4 and recentrifuged as before. The pellet from this centrifugation is resuspended in 10 mM HEPES-Tris and homogenized three times for 30 seconds each with the T185 shaft of the Tekmar at a setting of 5. The resulting homogenate is centrifuged for 20 minutes in the GSA rotor at 14,000 x *g*. The supernatant is then centrifuged in the GSA rotor at 27,500 x *g* for 70 minutes.

After the preliminary centrifugations the membranes are suspended in 50% sucrose, 150 mM KCl, 100 mM TrisCl, and 5 mM Na pyrophosphate. These vesicles are loaded onto the bottom of a four step discontinuous gradient with additional steps at 30%, 21.5%, and 9.5% sucrose. This gradient is centrifuged for 1.5 hour at 193,000 x *g* in a Beckman 50.2Ti rotor. The pellicle at the 9.5%-21.5% sucrose interface is enriched in surface sarcolemma. This pellicle is collected and diluted into a buffer containing 150 mM KCl, 0.8 mM $MgSO_4$ and 10 mM TrisCl (pH=7.4 @ 22°C), then centrifuged for 35 minutes at 193,000 x *g*. The resulting pellet is resuspended in loading buffer and centrifuged once again. The final

pellet is resuspended in loading buffer to a final protein concentration of 2 mg protein/ml, frozen in liquid N_2 and stored at -70°C until use.

Membrane vesicles (20-40 μg protein) are loaded with 150 mM KCl and diluted 50-fold into 1 ml of binding buffer containing 150 mM KCl. The vesicles are preincubated 5 minutes at 37°C and then incubated for an additional time required for the attainment of equilibration conditions (exact time determined by preliminary experiments) in the presence of varying concentrations of [^{125}I]Compound 1, 2 , 3, or 4 (1 nM-1 μM). The binding reaction is terminated by addition of 4 ml of ice cold binding buffer and then rapid filtration over Whatman GF/C filters followed by three additional 4 ml washes with ice cold binding buffer. The radioactivity associated with the filters is determined using standard gamma counting techniques. Specific [^{125}I]Compound 1, 2, 3, or 4 binding is defined as total binding minus binding measured in the presence of 1-10 μM cold Compound 1, 2, 3, or 4.

The above assay is validated using the procedures outlined in paragraphs (a) - (f) of Example 13.

Example 15: Recombinant Receptor Binding Assay

The following is one example of a rapid screening assay for useful compounds of this invention. In this assay, a cDNA or gene clone encoding the I_{to} channel binding site (receptor) from a suitable organism such as a human is obtained using standard procedures. Such receptors have been cloned and are known in the art. Distinct fragments of the clone are expressed in an appropriate expression vector to produce the smallest polypeptide(s) obtainable from the receptor which retain the ability to bind Compound 1, 2, 3, or 4. In this way, the polypeptide(s) which includes the novel Compound 1/

Compound 2/Compound 3/Compound 4 receptor for these compounds can be identified. Such experiments can be facilitated by utilizing a stably-transfected mammalian cell line (*e.g.*, HEK 293 cells) expressing the I_{to} channel.

Alternatively, the Compound 1/Compound 2/ Compound 3/Compound 4 receptor can be chemically reacted with chemically modified Compound 1, 2, 3, or 4 in such a way that amino acid residues of the Compound 1/Compound 2/Compound 3/Compound 4 peptide receptor which contact (or are adjacent to) the selected compound are modified and thereby identifiable. The fragment(s) of the Compound 1/Compound 2/Compound 3/Compound 4 receptor containing those amino acids which are determined to interact with Compound 1, 2, 3, or 4 and are sufficient for binding to said molecules, can then be recombinantly expressed, as described above, using a standard expression vector(s).

The recombinant polypeptide(s) having the desired binding properties can be bound to a solid phase support using standard chemical procedures. This solid phase, or affinity matrix, may then be contacted with Compound 1, 2, 3, or 4 to demonstrate that those compounds can bind to the column, and to identify conditions by which the compounds may be removed from the solid phase. This procedure may then be repeated using a large library of compounds to determine those compounds which are able to bind to the affinity matrix, and then can be released in a manner similar to Compound 1, 2, 3, or 4. However, alternative binding and release conditions may be utilized in order to obtain compounds capable of binding under conditions distinct from those used for Compound 1/ Compound 2/Compound 3/Compound 4 peptide binding (*e.g.*, conditions which better mimic physiological conditions encountered, especially in pathological states). Those compounds which do bind can thus be selected from a very large collection of compounds present in a liquid medium

or extract.

Once compounds able to bind to the Compound 1/ Compound 2/Compound 3/Compound 4 binding polypeptide(s) described above are identified, those compounds can then be readily tested in the various assays described above to determine whether they, or simple derivatives thereof, are useful compounds for therapeutic treatment of cardiac and neurological disorders described above.

In an alternate method, native Compound 1, 2, 3, or 4 receptor can be bound to a column or other solid phase support. Those compounds which are not competed off by reagents which bind other sites on the receptor can then be identified. Such compounds define novel binding sites on the receptor. Compounds which are competed off by other known compounds, thus bind to known sites, or bind to novel sites which overlap known binding sites. Regardless, such compounds may be structurally distinct from known compounds and thus may define novel chemical classes of agonists or antagonist which may be useful as therapeutics.

Example 16: Block of Kv4.2 Transient Outward K^+ Channels By Compounds 1, 2, and 4

Two types of transient outward K^+ channels have been cloned from rat heart cDNA libraries, Kv1.4 and Kv4.2 (Roeberds & Tankun, 1991, *Proc. Natl. Acad. Sci. USA*. 88: 1798-1802; Blair, et al., 1991, *FEBS Letters*. 295:211-213). The effects of Compounds 1 and 2 were evaluated on each of these two types of transient outward potassium channels expressed in *Xenopus* oocytes as described below. The effects of Compound 4 on Kv4.2 was also determined.

Isolation of oocytes and injection of RNA

Xenopus frogs were anesthetized by immersion in 0.2% tricaine for 15-30 min. Ovarian lobes were digested with 2 mg/ml Type 1A collagenase (Sigma) in Ca^{2+}-free ND96

solution for 1.5 hours to remove follicle cells. Stage IV and V oocytes (23) were injected with Kv4.2 or Kv1.4 cRNA (0.05 mg/ml, 50 nl), then cultured in Barth's solution supplemented with 50 μg/ml gentamycin and 1mM pyruvate at 18°C. Barth's solution contained (in mM): 88 NaCl, 1 KCl, 0.4 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 1 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES; pH 7.4.

Plasmids containing Kv1.4 and Kv4.2 cDNA were used, as described in Blair et al., 1991, *FEBS Letters* 295:211-213 and Po et al., 1992, *Circ. Res.* 71:732-736. Templates for cRNA synthesis from channel DNA were prepared as described by Po et al., 1992, *Circ. Res.* 71:732-736.

Two-microelectrode voltage clamp of oocytes

Oocytes were bathed in ND96 solution. This solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES; pH 7.6. Currents were recorded at room temperature (21-23°C) using standard two-microelectrode voltage clamp techniques. Glass microelectrodes were filled with 3 M KCl and their tips broken to obtain tip resistances of 1-3 MΩ for the voltage-recording electrode and 0.6-1 MΩ for the current-passing electrode. Oocytes were voltage-clamped with an Axoclamp 2A amplifier. Voltage commands were generated using pCLAMP software (ver. 5.5, Axon Instruments), a 486DX2 personal computer and a Digidata 1200 D/A interface (Axon Instruments). Current signals were digitally sampled at a rate equal to 2-4 times the low-pass cut-off frequency (-3 db) of a 8-pole Bessel filter. The oocyte membrane potential was held at -70 mV between test pulses, applied at a rate of 1-3 pulses/min.

The time course of Kv4.2 inactivation was fit with a single exponential relationship: $I(t) = A_0 + A_1 e^{(-t/\tau)}$, using a Chebyshev non-iterative fitting technique (pCLAMP, Axon Instruments). The voltage dependence of Kv4.2

inactivation was determined using 8 sec conditioning test pulses, applied once every 30 sec. Each conditioning pulse was applied to a potential ranging from -120 mV to 0 mV, and was followed by a pulse to +40 or +75 mV to monitor the extent of channel inactivation.

Data analyses, including exponential fitting of current traces, were performed using pCLAMP. Fits of approriate data to a Boltzmann function or Hill equation were performed using Kaleidagraph (Synergy Software). Data are expressed as the mean ± SEM.

Specific Block of Kv4.2 Channels

As noted above, two types of transient outward K^+ channels have been cloned from rat heart cDNA libraries, Kv1.4 and Kv4.2. The effects of the toxins on these two cloned K^+ channels expressed in *Xenopus* oocytes was tested. Compound 1 and Compound 2, at a concentration of 2 μM, had no effect on Kv1.4. All three compounds blocked Kv4.2 in a concentration-dependent manner. For example, at a test potential of -5 mV, Compound 2 decreased Kv4.2 84% at 400 nM, 72% at 200 nM, and 58% at 67 nM (n=4). The voltage-dependence of block of Kv4.2 was determined using a concentration of toxin that decreased current by 50% at a test potential near 0 mV (100 nM for Compound 4 and Compound 1, and 67 nM for Compound 2) (See Figs. 6 and 7). The voltage-dependence of block was linear for 100 nM Compound 4 and 67 nM Compound 2 (Fig. 6), but was curvilinear for 100 nM Compound 1 (Fig. 7). The data for Compound 1 was well described with the sum of a linear component and a sigmoidal component fit with a Boltzmann function. From these data, the voltage at which Kv4.2 was blocked by 50% was determined to be +4 mV for all three compounds.

Formulation and Administration

As demonstrated herein, useful compounds of this

invention and their pharmaceutically acceptable salts may be used to treat neurological disorders or diseases. While these compounds will typically be used in therapy for human patients, they may also be used to treat similar or identical diseases in other vertebrates such as other primates, farm animals such as swine, cattle and poultry, and sports animals and pets such as horses, dogs and cats.

In therapeutic and/or diagnostic applications, the compounds of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton PA.

Pharmaceutically acceptable salts are generally well known to those of ordinary skill in the art, and may include, by way of example but not limitation, acetate, benzenesulfonate, besylate, benzoate, bicarbonate, bitartrate, calcium edetate, camsylate, carbonate, citrate, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, mandelate, mesylate, mucate, napsylate, nitrate, pamoate (embonate), pantothenate, phosphate/disphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, or teoclate. Other pharmaceutically acceptable salts may be found in, for example, *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA (18th ed, 1990).

Preferred pharmaceutically acceptable salts include, for example, acetate, benzoate, bromide, carbonate, citrate, gluconate, hydrobromide, hydrochloride, maleate, mesylate, napsylate pamoate (embonate), phosphate, salicylate, succinate, sulfate, or

tartrate.

The useful compounds of this invention may also be in the form of pharmaceutically acceptable complexes. Pharmaceutically acceptable complexes are known to those of ordinary skill in the art and include, by way of example but not limitation, 8-chlorotheophyllinate (teoclate).

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.*, Fingl et al., in *The Pharmacological Basis of Therapeutics*, 1975, Ch. 1 p. 1).

It should be noted that the attending physician would know how and when to terminate, interrupt, or adjust administration due to toxicity or organ dysfunction. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical responses were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated into liquid or solid dosage forms and administered systemically or locally. Techniques for formulation and administration may be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA. Suitable routes may include oral, buccal, sublingual, rectal, transdermal, vaginal,

transmucosal, nasal or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical

lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspension. Suitable lipophilic solvents or vehicles include fatty oils such as

sesame oil, or synthetic fatty acid ester, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose (CMC), and/or polyvinylpyrrolidone (PVP: povidone). If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol (PEG), and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dye-stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well

as soft, sealed capsules made of gelatin, and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols (PEGs). In addition, stabilizers may be added.

For topical administration, the compounds of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

The amount of various compounds of this invention which should be administered can be determined by standard procedures.

Other embodiments are within the following claims.

Claims

What is claimed is:

1. A specific potent transient outward potassium channel inhibitor.

2. The inhibitor of claim 1, wherein said inhibitor is selected from the group consisting of Compound 1 SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), Compound 3 (SEQ ID NO. 3), and Compound 4 (SEQ ID NO. 4).

3. An inhibitor obtainable from a spider venom, or a unique fragment or analog of said polypeptide active to modulate a potassium channel.

4. A method for screening for a transient outward potassium channel active agent, comprising the steps of:

contacting a transient outward potassium channel with a known specific transient outward potassium channel inhibitor which binds to said transient outward potassium channel and a potential transient outward potassium channel active agent, and

detecting inhibition of binding of said known specific transient outward potassium channel inhibitor to said transient outward potassium channel by said potential transient outward potassium channel active agent, wherein said inhibition of binding is indicative of a useful transient outward potassium channel active agent.

5. The method of claim 4 wherein said known specific transient outward potassium channel inhibitor is selected from the group consisting of Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), Compound 3 (SEQ ID NO.

3), and Compound 4 (SEQ ID NO. 4).

6. The method of claim 4 wherein said transient outward potassium channels are from cardiac or neural tissue.

7. The method of claim 4, wherein said known specific transient outward potassium channel inhibitor is active on a subset of types of transient outward potassium channels.

8. A method for screening for an agent active on a Kv4.2 or Kv4.3 potassium channel, comprising the steps of:

contacting a Kv4.2 or Kv4.3 potassium channel with a known specific transient outward potassium channel inhibitor active on said Kv4.2 or Kv4.3 potassium channel and a potential agent active on said channel, and

detecting inhibition of binding of said known specific transient outward potassium channel inhibitor by said potential agent active on said channel,

wherein inhibition of binding is indicative of a useful agent active on said channel.

9. The method of claim 8, wherein said Kv4.2 or Kv4.3 potassium channels are expressed in oocytes.

10. The method of claim 8, wherein said transient outward potassium channel active agent is not active on a Kv1.4 channel.

11. The method of claim 10, wherein said known specific transient outward potassium channel inhibitor is selected from the group consisting of Compound 1 (SEQ ID NO. 1) and Compound 2 (SEQ ID NO. 2).

12. A method for screening for a potassium channel active agent, comprising the steps of:

contacting a potassium channel with a known potassium channel inhibitor which binds to said potassium channel and a potential potassium channel active agent, wherein said known potassium channel inhibitor is derived from spider venom, and

detecting inhibition of binding of said known potassium channel inhibitor to said potassium channel by said potential potassium channel active agent, wherein said inhibition of binding is indicative of a useful potassium channel active agent.

13. A method for treatment of a disease or condition in which modulation of transient outward potassium channel activity is therapeutically useful, comprising the step of:

administering a therapeutically effective specific transient outward potassium channel inhibitor.

14. The method of claim 13, wherein said specific transient outward potassium channel inhibitor corresponds to an inhibitor present in a spider toxin.

15. The method of claim 14, wherein said transient outward potassium channel inhibitor is selected from the group consisting of Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), Compound 3 (SEQ ID NO. 3), and Compound 4 (SEQ ID NO. 4).

16. The method of claim 13, wherein said specific transient outward potassium channel inhibitor is active on a Kv4.2 or Kv4.3 channel.

17. A method for treatment of a disease or

condition in which modulation of potassium channel activity is therapeutically useful, comprising the step of:

administering a therapeutically effective potassium channel inhibitor corresponding to an inhibitor present in a spider toxin.

18. A pharmaceutically acceptable composition comprising a compound selected from the group consisting of Compound 1 (SEQ ID NO. 1), Compound 2 (SEQ ID NO. 2), Compound 3 (SEQ ID NO. 3), and Compound 4 (SEQ ID NO. 4).

19. A method for the use of a potassium channel inhibitor isolated from spider venoms as an insecticidal agent, comprising the step of:

applying to an insect or its environment an inhibitor present in spider venom.

FIG. 1

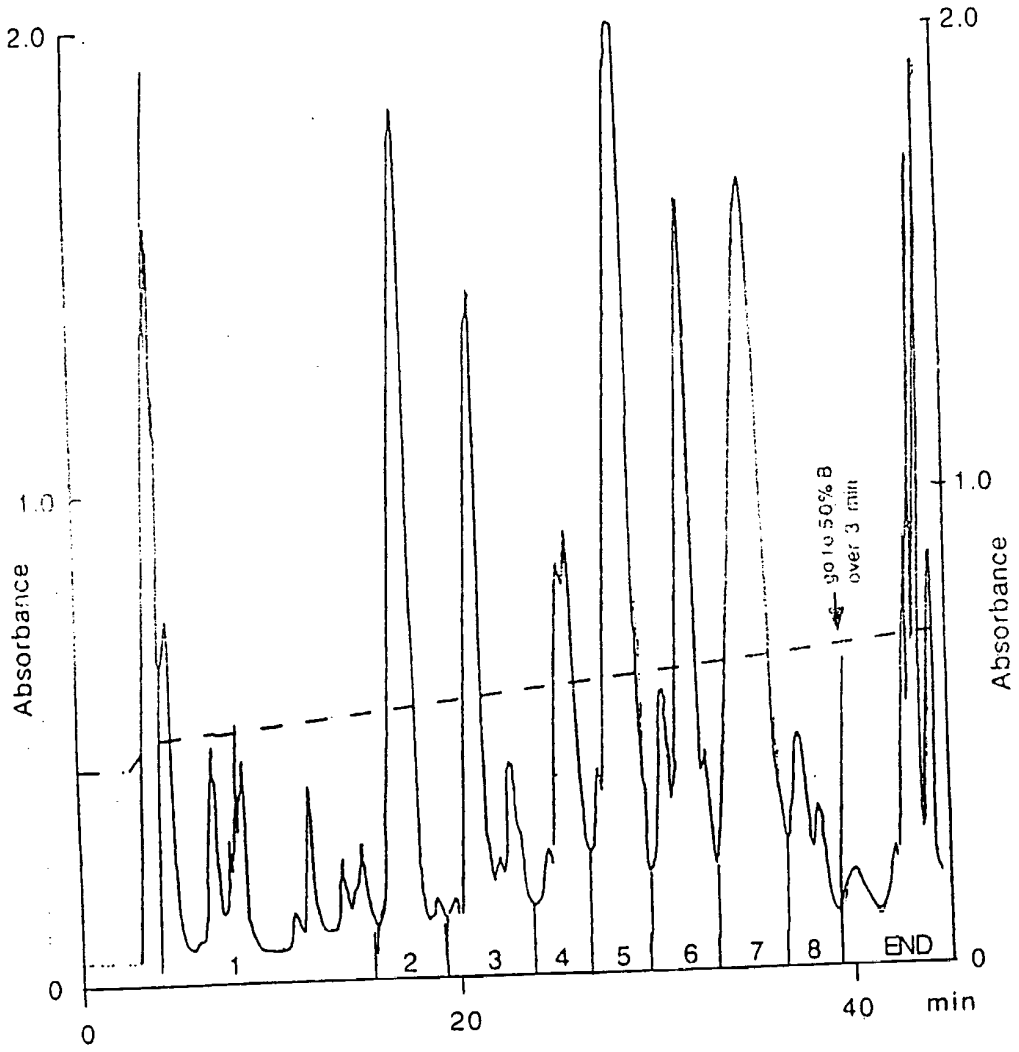

2.0
1.0
0
Absorbance
2.0
1.0
0
Absorbance
go to 50% B
over 3 min
1
2
3
4
5
6
7
8
END
0
20
40
min

FIG. 2

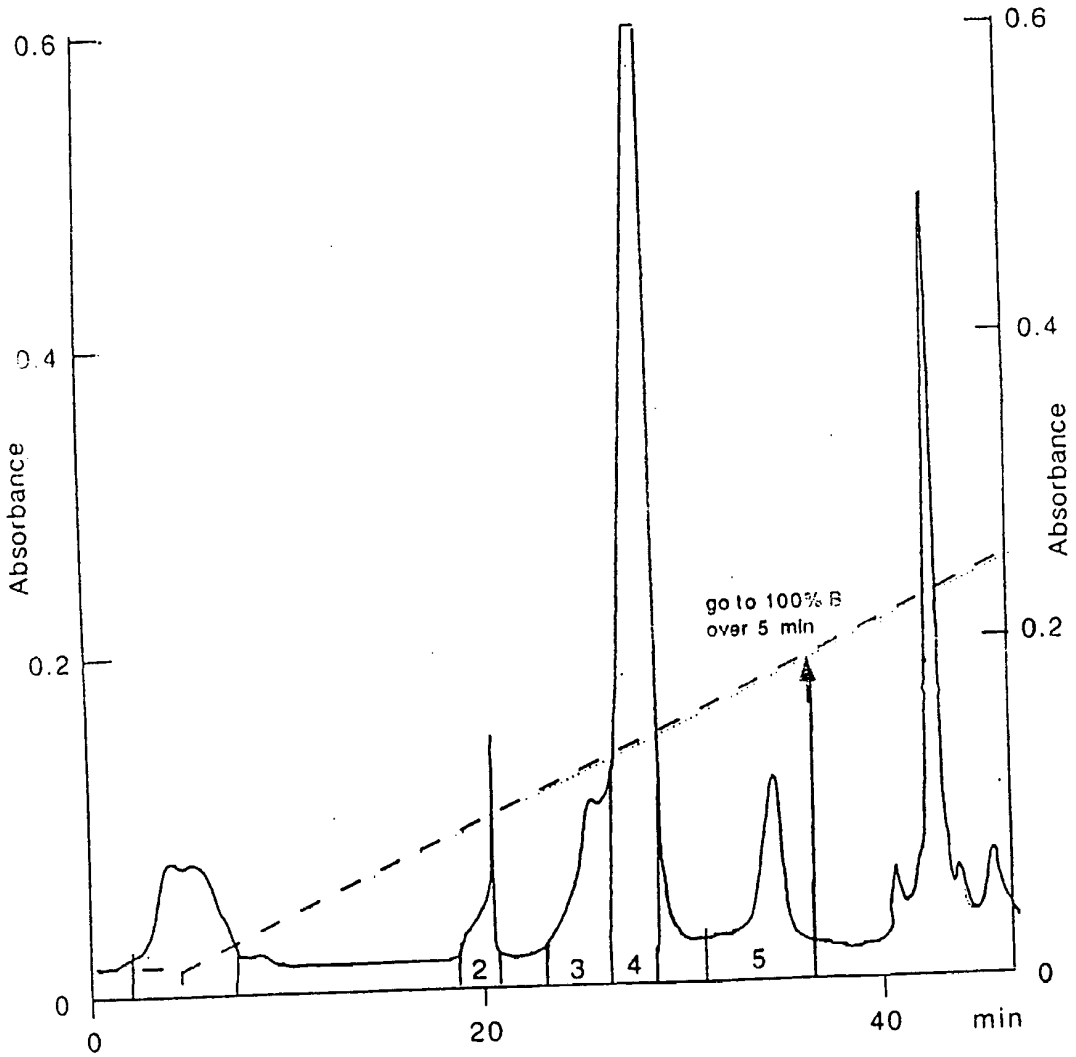

0.6
0.4
0.2
0
Absorbance
go to 100% B
over 5 min
1
2
3
4
5
0
20
40
min
0.6
0.4
0.2
0
Absorbance

FIG. 3

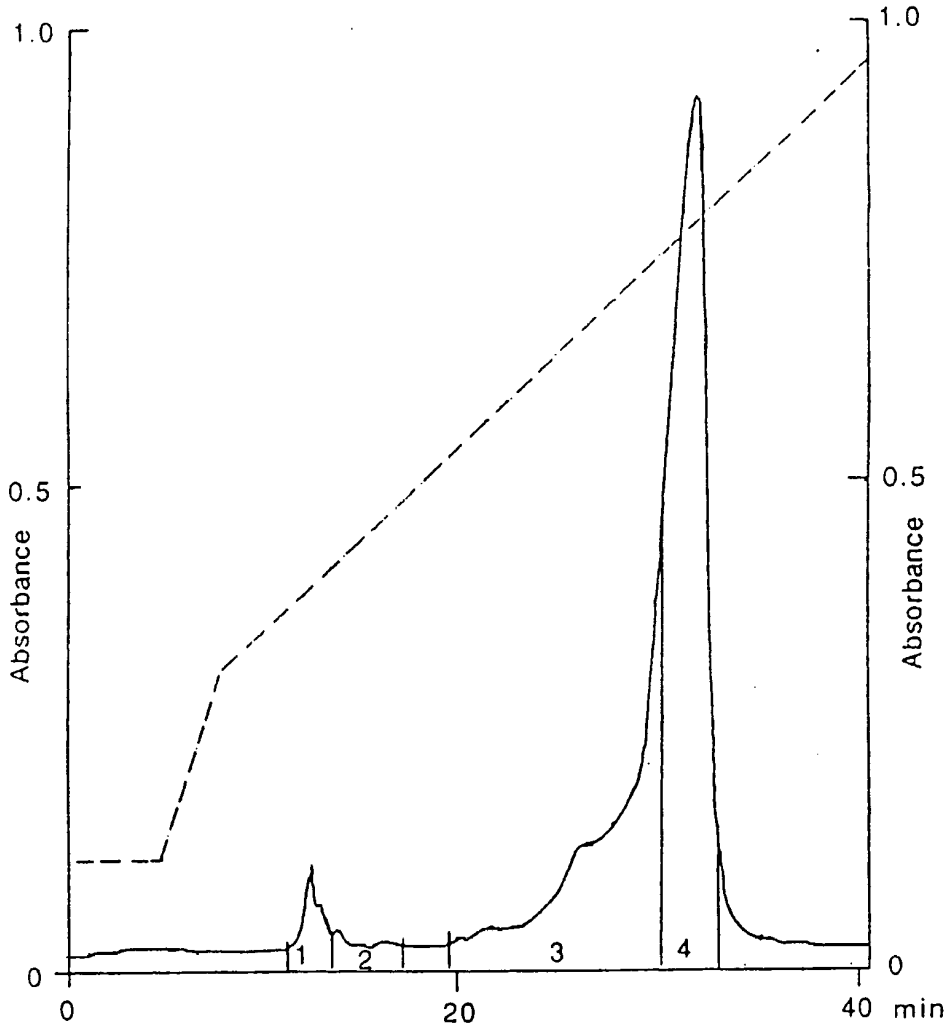


FIG. 4

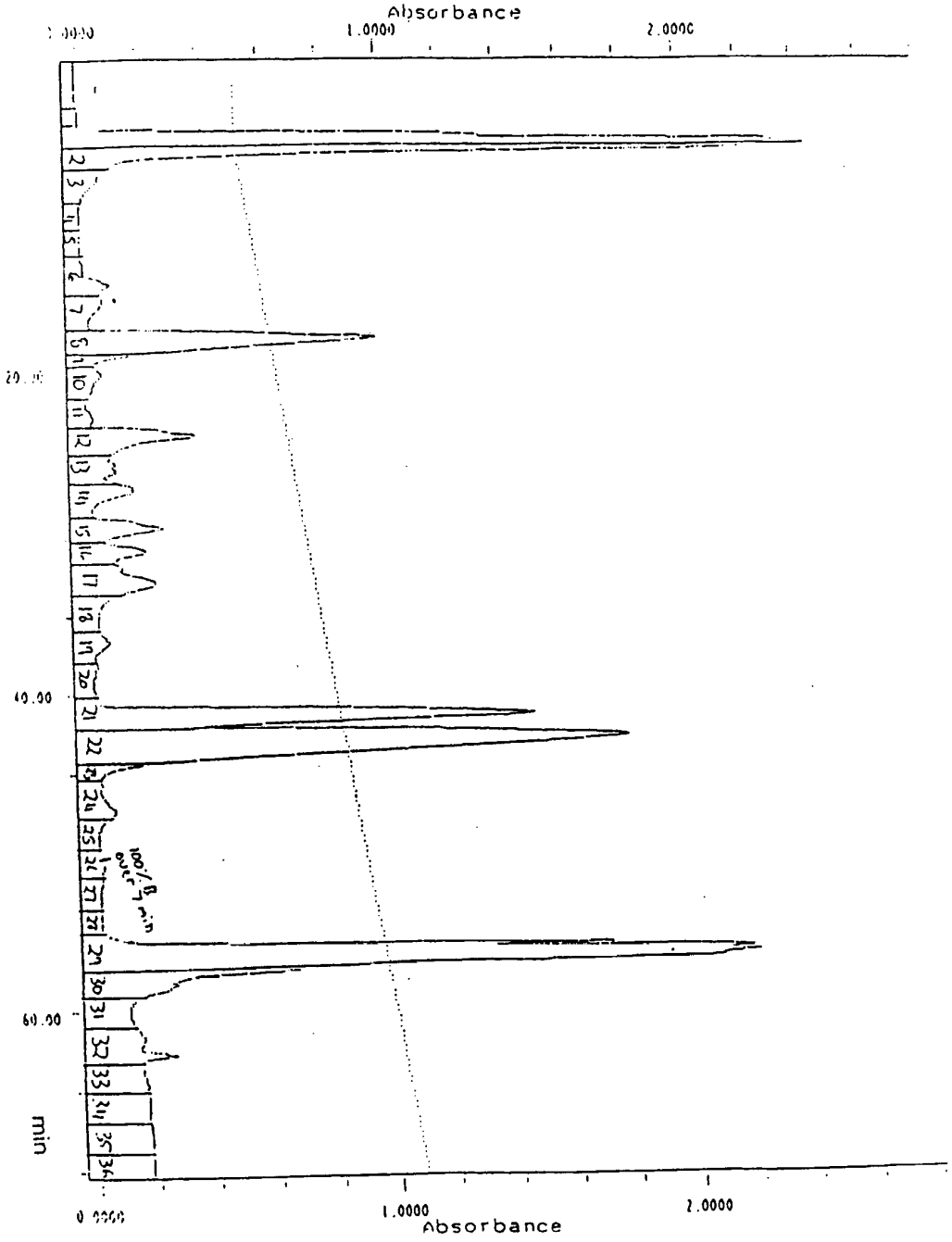

Absorbance
1.0000
2.0000
40.00
60.00
min
0.0000
1.0000
2.0000
Absorbance

FIG. 5

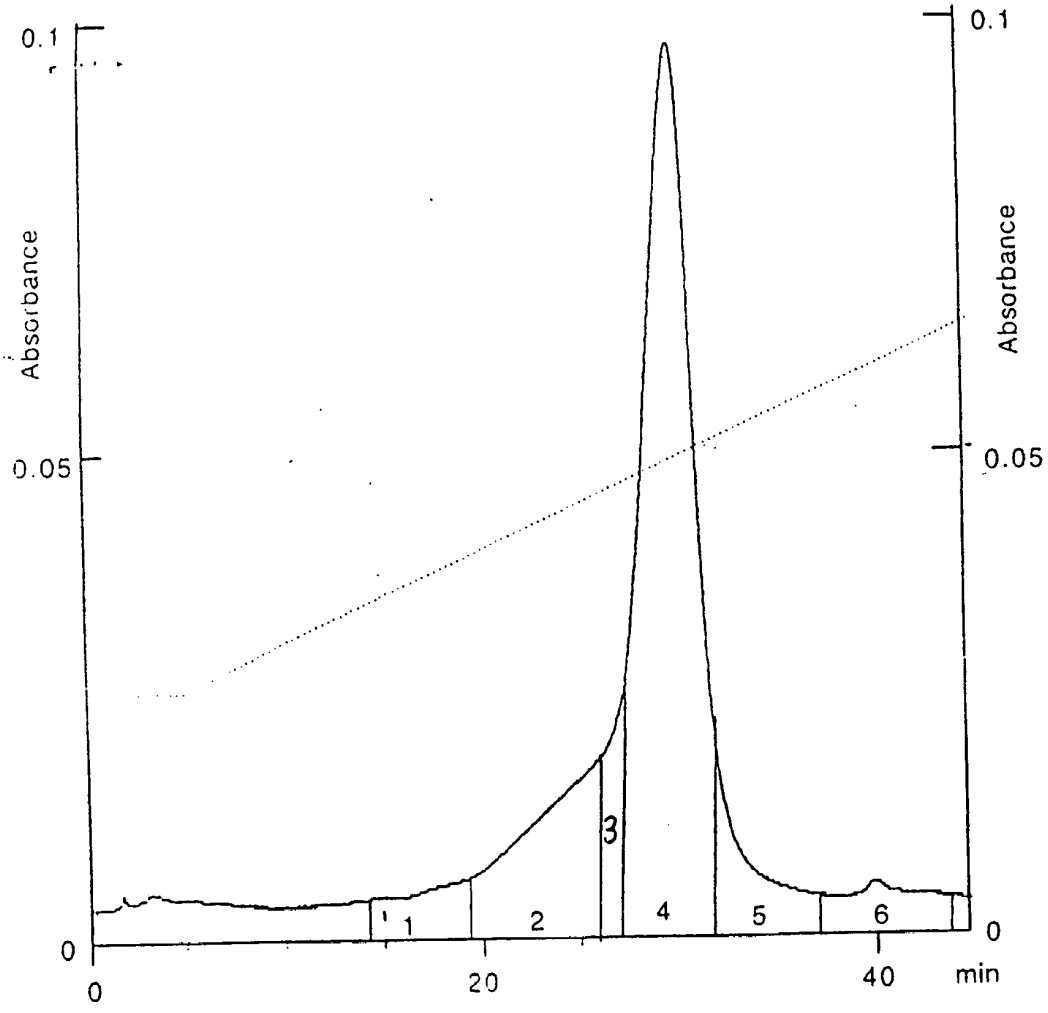


FIG. 6

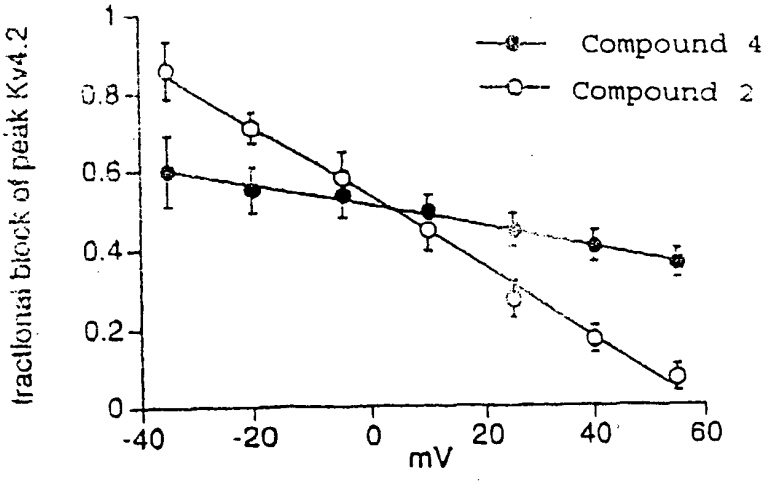
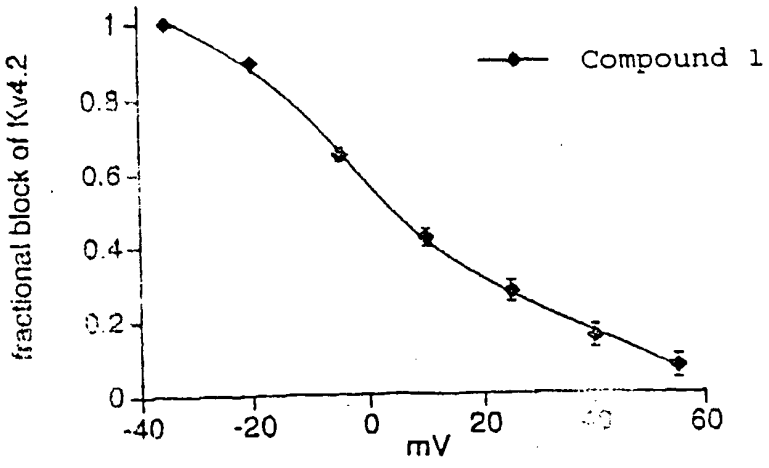

fractional block of peak Kv4.2
1
0.8
0.6
0.4
0.2
0
-40
-20
0
20
40
60
mV
Compound 4
Compound 2

FIG. 7


fractional block of Kv4.2
1
0.8
0.6
0.4
0.2
0
-40
-20
0
20
60
mV
Compound 1